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STUDIES ON THE POTENTIAL OF RADIOLABELED MAb174H64 IN THE DETECTION AND RADIOIMMUNOSCINTIGRAPHY OF SQUAMOUS CELL CARCINOMA (SCC) AND THE PRELIMINARY CHARACTERIZATION OF THE SCC ASSOCIATED ANTIGEN

BY

FASSIL HAILU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES (RADIOPHARMACY)

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

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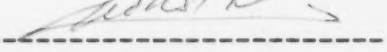

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I dedicate this

To my wife Tadelech Asfaw and to my children Michael, Helen and Hirut who sacrificed their interest to support me in my studies

ABSTRACT

Squamous cell carcinoma (SCC) is the predominant type of tumor of the head & neck, lungs, oral cavity and cervix. Previous research conducted to develop a sensitive diagnostic method for an early detection of the tumor were not successful. A monoclonal antibody MAb174H.64, implicated to have immunoreactivity with SCC tissues, was investigated to determine the SCC antigen responsible for the immunoreaction. Cytokeratin proteins were extracted from bovine squamous cell carcinoma tissue and SCC cell lines (FaDu and KLN-205). ELISA results showed that MAb174H64 has specific immunoreactivity with the protein extracts of the above cells and tissues. Results from SDS-PAGE electrophoresis showed that the cytokeratin protein dimer is composed of two monomers with molecular size of 58 kd and 64 kd, corresponding to Molls' catalogue # 10 and # 2 or 3 respectively.

The results of a 24 hr biodistribution study in Balb/c mice bearing a KLN-205 allograft, injected i.v. with a mixture of ^{99m}Tc174H.64 and ^{125}I-MOPC-21 (control), showed that the tumor-to-blood ratio of ^{99m}Tc174H.64 was twice as much as that of ^{125}I-MOPC-21. DBA mice bearing a KLN-205 allograft were injected i.v. with a mixture of ^{125}I-174H64 and ^{131}I-MOPC-21 (control) and a biodistribution study was performed at 6 hr, 24 hr and 48 hr post-injection time. The results showed increased tumor-to-blood ratios of ^{125}I-174H64 with

elapsed time. At post-injection times of 24 and 48 hours, the tumor-to-blood ratios of ^{125}I-174H64 was significantly more than that of ^{131}I-MOPC-21 ($p < 0.025$).

A 24 hr whole-body scintigraphic study of a Balb/c mouse, with a FaDu SCC xenograft, injected i.v. with ^{99m}Tc-174H64, showed a distinct localization of the radiolabeled antibody in the tumor. Whole-body scintigraphy of a DBA mouse, bearing a KLN-205 allograft, injected i.v. with ^{125}I-174H64 was performed at 6 hr, 24 hr, 48 hr and 120 hr post-injection times. The results showed a distinct localization of radioactivity in the tumor.

These results suggest that SCC tissues have tumor associated cytokeratin protein antigens and that MAb174H64 is specifically immunoreactive with the cytokeratin proteins. MAb174H64 labeled with an appropriate radioisotope has a promising potential to be useful in the diagnosis and possibly in the treatment of malignant SCC tumor. Quantitative isolation of the cytokeratin protein monomers from their dimer forms, is recommended for quality control process. Further clinical studies are also recommended to refine the MAb174H64 applicability for human use.

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LIST OF ABBREVIATIONS

ABC reagent	avidin-biotin peroxidase conjugated
ABTS	2,2,-azino-di(3-ethylbenzthiazoline sulphonate
AFP	alphafetoprotein
AOI	area of interest
BF	bound fraction
BFC	bifunctional chelating agent
BSA	bovine serum albumin
CEA	carcinoembroyonic antigen
CE-DTS	p-carboxylphenyloxal-di-(N-methylthio semicarbazone
CPM	counts per minute
CPS	counts per second
CT	computed tomography
N_2S_2	diamino dimercaptile
DDT	dithiothreitol
DEAE	diethylene diaminoethyl
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
$F(ab')_2$	antibody binding fraction (region)
Fc	the constant region of antibody
FCS	fetal calf serum

Fv	variable region of IgG
Gy	gray
GBq	gigabecquerel
IgG	gamma immunoglobulin
HAT	hypoxanthine, aminopterine, thymidine
HCl	hydrogen chloride
hr	hour
HPHT	high performance hydroxyl apatite
HPLC	high pressure liquid chromatography
ICI	iodine monochloride
i.p.	intraperitoneal
i.v.	intravenous
I.F.	intermediate filament
IRU	immuno reactivity unit
kBq	kilobecquerel(s)
kd	kilo dalton
L	liter(s)
M	molar
MAb	monoclonal antibody
MBq	megabecquerel
mCi	millicurie
MTS	Michegan terminal systemof main frame computing system
μCi	microcurie
mg	milligram(s)

μg	microgram(s)
μL	microliter(s)
min.	minute(s)
mL	milliliter(s)
mM	millimole(s)
MAC	methyl amino-propyl cyclam
MN antigens	the major antigens of the second human blood group system
MRI	magnetic resonance imaging
N	normal
OD	optical density
PBS	phosphate buffered saline
PEG	polyethylene glycol
PGP	phosphoglycoprotein
NaCl	sodium chloride
NaOH	sodium hydroxide
Rf	ratio of distance traveled by the sample to that of the solvent front
SDS-PAGE	sodium-dodecile sulfate-polyacrilamide gel electrophoresis
SCC	squamous cell carcinoma
sFv-RP	recombinant single chain Fv
sFv	single chain Fv
S.D.	standard deviation
TCA	trichloroacetic acid
T-antigen	Thomsen-Friedenreich antigen

Tn	β-galactosidase treated T-antigen
TcO_4^-	pertechnetate ion
TLC	thin layer chromatography
TSA	tumor specific antigen
TAA	tumor associated antigen
TTHA	triethylenetetraaminehexaacetic acid
UBF	unbound fraction
uv	ultraviolet

1. INTRODUCTION

The application of radioisotopes in the diagnosis and treatment of various disorders in humans has been increasing in the last decade. The first attempt to use a radioisotope as a diagnostic agent for human use was performed in 1926 by Blumgart, Yens and Weiss (Anderson G.A., 1976). They determined the arm-to-arm circulation time in a patient by injecting a few millicuries of radium C intravenously in one arm and detecting its arrival in the other arm, using a simple Wilson Cloud Chamber detector. Since then many startling discoveries have been made in the development of new artificially produced radioisotopes with diverse properties. Along with the development of new techniques of artificially producing various radioactive elements, the concept of attaching radioisotopes to biologically useful compounds expanded greatly.

Biological molecules which have the unique property of localizing at a specific site of importance in the body have been studied for their potential application in the investigation and treatment of cancer. One of the most important discoveries made in this field was the employment of antibodies made against malignant tissues for cancer treatment. The pioneering work of antibody radiolabeling and tumor localization was conducted by Pressman and co-workers in 1948-1953 (Pressman D., Hill R.F. and Foote F.W., 1948; Pressman D. and Keighly G., 1948; Pressman D., Eisen H.N. and Sternberger L.A., 1949; Pressman D. and Korngold L., 1953).

Subsequently they were able to demonstrate that radioiodinated antibodies could be used for the detection of tumor. Bale *et al.* showed that antibodies localized in tumor can be used to carry a therapeutic dose of radioactivity to the tumor (Bale W.F., *et al*, 1960). Since then many researchers have used radiolabeled anti-tumor antibodies for the detection and treatment of tumors (Williams M.R., *et al.*, 1984; Barbaian R.J., *et al.*, 1987; Perkins A.C., *et al.*, 1991a,b,c; McEwan A.J.B. *et al.*, 1992). Although antibodies showed potential in this field, the problem of non-specific localization and cross reactivity overshadowed their usefulness. Therefore the need for more specific anti-tumor antibodies became obvious. The hybridoma technology which made the production of specific antibodies possible was developed in 1975 by two Nobel laureates, G. Köhler and C. Milstein. The availability of tumor specific monoclonal antibodies stimulated a great interest in the pursuit of the use of radiolabeled antibodies for tumor imaging (Köhler G. and Milstein C., 1975).

The investigation and utilization of these two major breakthrough technologies for the development of a useful diagnostic agent is the goal of this thesis work.

The main objective of the experiments presented in this thesis was to evaluate the potential use of radiolabeled MAb-174H.64 for the diagnosis of squamous cell carcinoma. In order to evaluate the specificity of the antibody to the tumor, the antigen responsible for this reaction was

extracted, isolated and characterized. This antigen was subsequently used to evaluate the immunological and radiochemical stabilities of radiolabeled MAb174H.64.

The relative localization of radiolabeled MAb174H.64 in two animal models bearing a SCC tumor was evaluated. The clearance of radioactivity from the whole-body and the various critical organs of the animal models was determined. The blood clearance of the radiolabeled whole-antibody and its $F(ab')_2$ fragment were compared. Based on the results obtained from these experiments the prospect of the use of radiolabeled MAb174H.64 in the radioimmunodetection (RAIS) of SCC tumor was discussed.

2. SURVEY OF THE RELATED LITERATURE .

2.1. TUMOR DETECTION.

Tumor cells are transformed cells that behave in a rather abnormal way different from the cells of origin. These changes are manifested by a number of characteristics. The increased rate of proliferation, altered growth requirements, loss of contact inhibition, ability to metastasize and altered cell surface compositions (Liotta L.A., 1986) are some of the criteria on which tumor detection are based.

The localization of radiolabeled tracers in tumor provides a non-invasive method of detection, a proficient way for treatment and an effective means of assessment and follow up of treatment procedures (Paterson A.H.G. and McReady V.R., 1975). In the following sections the basic concepts leading to the various approaches in the detection of tumors are reviewed.

2.1.1. Conventional Methods of Tumor Detection.

The conventional method of tumor detection can be classified into three sub-classes. These are 1) External tumor detection methods. 2) Indirect tumor localization method and 3) Direct tumor localization method.

2.1.1.1 The external method of tumor detection.

The external method of tumor detection can be summarized as following. The first method is the physical examination of the size and number of superficially localized tumor nodes. In this method the clinical assessment of the tumor is based

on palpation (Sako K. *et al.*, 1964; Vikram B., 1984) and it can be useful only for the determination of the number and size of involved nodes, in which case it must be externally palpable. The second method of tumor detection is based on subjective microscopic and macroscopic evaluation, which is now modernized by the introduction of electron microscopy and histochemistry. This method requires biopsy samples. The selection of the biopsy site and the technique of removal of samples is very critical to the detection process (Robbins S.L. *et al.*, 1981). The risk of spreading a potentially metastatic tumor during the biopsy process can be very high (Raz A. and Lotan R., 1987). The third method of external detection of tumor is based on taking images of the tumor utilizing new technology including ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). These modern imaging techniques have the advantages over the other external detection methods, because they are not invasive as those which require biopsy, they are objective and they provide a permanent record. However, their capability for detecting small nodes is limited and very often, like the palpation method, they cannot discriminate between normal nodes, reactively enlarged nodes and tumor infiltrated nodes.

2.1.1.2 Indirect tumor localization:

Tumor cells function abnormally. Some of their functional changes or the damage they cause can be exploited to differentiate them from the surrounding normal cells. This may lead to a lack of uptake of some radiolabeled compounds or radionuclides (Lopaz-Majona V. and Alvarez-Crevera J., 1979). Examples of the common cases are:

1) Liver cancer that causes damage to parenchymal tissues can be seen as a "cold" spot when scanned after i.v. injection of ^{99m}Tc-sulfur colloid.

2) Brain tumor, which causes damage to the blood brain barrier can be detected by the presence of a "hot" spot upon the administration of ^{99m}Tc-sodium pertechnetate as an indication of damage to the barrier.

3) A "cold" spot is also observed in non-functional thyroid tumor tissue when sodium ^{131}I is administered, while the normally functioning cells show "hot" area.

The disadvantages of these agents are their non-specificity in tumor localization and the limited capacity of using one radiopharmaceutical for one type of tissue only (Winch H.S., 1976).

2.1.1.3. Direct tumor localization.

The mechanism of detection of tumor by this method is based on the principle of localization of radiopharmaceuticals or radionuclides in the tumor tissues. These localizations may be achieved by conditions such as 1) increased metabolism in the tumor tissue with increased

uptake of radioactive metabolites, for example, ^{75}Se selenomethionine (Tubis M., 1976); 2) increased uptake of radionuclide due to a hyper-functioning organ like hyper-active thyroid gland, for example, Na ^{131}I (Siemsen J.K. and Telfer N., 1976); 3) the use of antibiotic anti-tumor agents, like bleomycin, adriamycin and tetracycline radiolabeled with a number of radioisotopes (Taylor D.M. and McReady V.R., 1976) and 4) the utilization of tumor-seeking radioisotopes such as ^{67}Ga citrate which was used as a soft tissue, bone and tumor localizing radionuclide (Beckerman C. *et al.*, 1985). Both ^{67}Ga citrate and bleomycin localize in non-malignant processes such as infection and inflammation and therefore are not specific to tumor detection.

All of the approaches discussed in the conventional method of tumor detection lack the important qualities of specificity and adequate sensitivity to detect small tumors. Early diagnosis of the tumor is very important to eradicating the disease while it is still small and localized. The conventional method cannot specifically identify this disease. Therefore, it is very important to have a means by which an early diagnosis of the tumor is achievable.

A more specific tumor detection approach based on the use of radiolabeled antibodies was first demonstrated by Pressman and Korngold (Pressman D. and Korngold L., 1953). Since then significant progress has been made to improve such properties as specificity, avidity and stability of radiolabeled antibodies. A review of the development and

utilization of radiolabeled antibodies is presented in the following section.

2.1.2. Monoclonal Antibodies in Tumor Detection.

The prospect of monoclonal antibodies in tumor detection is based on the assumption that tumors possess specific antigens (TSA), which are found exclusively in tumor but not in normal tissues. This condition was suggested to be observed only in experimentally induced tumors (Prehn R.T., 1989). However, in most reported cases of spontaneous tumors, TSA is not present, instead tumor associated antigens (TAA) were present in a concentration greater than that present in normal tissues (Damjanov I. and Knowless B.B., 1983). Monoclonal antibodies raised against tumor associated markers have been found to have a potential utility for tumor detection. The importance of utilization of a number of tumor associate markers for the detection of different types of neoplasm was the subject of a review by Zalcberg and McKenzie, (Zalcberg J.R. and McKenzie F.C., 1985).

Monoclonal antibodies labeled with suitable radio-isotopes and specifically immunoreactive with tumor associated antigens are very useful in the diagnosis of tumors *in vitro* as well as *in vivo*. However, there are many factors that affect the localization of radiolabeled monoclonal antibodies in tumor tissues. Some of these factors are summarized in the following paragraphs.

The path through which an intravenously injected radiolabeled monoclonal antibody travels before it reaches

its destination includes, extravasation (Cobb L.M., 1989), transport through the extra-cellular space of tissues (Jain R.K. and Wei J., 1977), access to the tumor cell (Moll R. *et al.*, 1986) and finally intratumoral antigen-antibody binding (Pervez S. *et al.*, 1988). Each of these pathways contributes to the obstacles observed in the *in vivo* localization of radiolabeled monoclonal antibodies. During extravasation the barrier created by normal capillary endothelial cell varies greatly between tissues. This barrier is defective in tumor tissues and causes an increase in the permeability of the capillaries (Jain R.K., 1985). In order for the antibodies to reach the majority of tumor cells it is very important to have extensive vascularisation in tumor tissues. After extravasation, antibodies will be in the extra-cellular space and move through this space by diffusion and convection (Swabb E.A. *et al.*, 1974). However, this mechanism will be severely hampered in tumor tissues and this prevents transportation of antibodies through the tissue space (Sweet M.B.E. *et al.*, 1979). As tumors start to grow, the increase in the interstitial pressure causes a reduced blood flow and finally causes central necrosis of the tumor mass, making it difficult for the antibody to reach tumor tissues at the center.

Once the antibodies have reached the peripheral cell layer of a tumor mass the only barriers that they have to pass in order to react with the antigen is the intracellular junction. These junctions are formed of desmosomes, in case

of normal squamous cell epithelium. However squamous cell carcinoma especially the poorly differentiated types, have fewer desmosomes than normal squamous cell epithelium (Moll R. *et al.*, 1986), and this improves the passage of antibodies into the tumor.

The intra-tumoral antigen-antibody binding can be affected by factors such as the proportion of antigen-positive cells, tumor vascularization, the presence of circulating antigens, and the accessibility of the antigenic sites. The size of the immunoglobulin has been shown to have an effect on its penetration through the barriers described in the preceding paragraph. The immunoglobulin fragments, $F(ab')_2$ and F(ab), are more penetrating than the intact whole-antibody (Matzku S. *et al*, 1988) and they also minimize the development of anti-mouse antibody response observed when intact antibody is used (Bates S.E. and Lango D.L., 1987; Hale G. *et al.*, 1988).

There are problems observed in the use of radiolabeled monoclonal antibodies for the detection of tumors. Problems arise from the damage caused by the radiolabeling process resulting in an altered biological activity of antibodies. The damage to the immunologically sensitive area leads to non-specific localization of the radiolabeled antibody (Keenan A.M. *et al.*, 1985). The non-specific localization of antibody can occur from molecular damage caused at any stage of the preparation. Cross reactivity with non-target antigen on the tumor surface can result in non-selective antibody

binding. The Fc region of the antibody non-specifically reacts with Fc receptors on some cells and the resulting antigen-antibody complex may be removed by the liver cells and thus contribute to the non-specific uptake by liver and cause a high background of radioactivity.

Many of these problems can be overcome by improving labeling and purification techniques and by developing more sensitive and specific monoclonal antibodies (Keenan A.M. *et al.*, 1985). The high non-target background radioactivity present in the blood pool, liver and spleen, which can occur as a result of all of the problems mentioned above, can be reduced. The approaches currently being investigated are presented in the following paragraphs (Goodwin D.A., *et al.*, 1986; Yuanfang L. and Chuanchu W., 1991).

1) The utilization of antibody fragments: Whole-antibody is cleared very slowly from the blood, resulting in high radioactivity in circulation. Antibody fragments such as Fab, Fab' and $F(ab')_2$, on the other hand, disappear from the blood into the extravascular compartment at a faster rate than the whole-antibody. Furthermore, Fab fragments rapidly clear from the blood as compared to the larger $F(ab')_2$ fragments. Results reported by both Wilbanks and Wahl indicated that tumor-to-blood ratio for the three forms of antibody have values of Fab > $F(ab')_2$ > Whole-antibody (Wilbanks A.B., *et al.* 1981 and Wahl R.L., *et al.*, 1983). Although the smaller fragments show higher tumor-to-blood ratio the absolute concentration of the antibody localized in the tumor is

maximum when the whole antibody is used. Wahl, using monoclonal anti-CEA whole antibody, $F(ab')_2$ and Fab fragments demonstrated that the absolute concentration of the whole-antibody is 2.4 times the $F(ab')_2$ concentration and 26 times the Fab concentration at 48 hr. In this case the $F(ab')_2$ fragment was the best compromise between the rapidly cleared Fab fragments and the slowly cleared whole-antibody.

2) The use of a second antibody: A second antibody, prepared using the first antibody as an antigen, is effective in lowering the blood background by interacting with the circulating (first) antibody. The resulting complex will be removed by the endothelial cells present in the liver. A disadvantage of this method is the concomitant rise in the liver background (Begent R.H.J. *et al.*, 1982).

3) The attachment of a metabolizable linker: An easily metabolizable compound used to link the antibody with the radioisotope has been shown to increase target to non-target ratio by lowering the blood background (Haseman M.K., *et al.*, 1986; Meares C.F., *et al.*, 1988 and Quadri S.M. *et al.*, 1986).

4) Local delivery: This involves injecting the antibody directly at the site were the tumor is located and therefore is limited to accessible areas (Courtnay-Luck N., *et al.*, 1984).

5) Pre-targeted immunoscintigraphy: In this case non-radioactive-antibody is administered first and allowed to reach a maximum tumor concentration in about a day. Then the

excess antibody circulating in the blood is cleared by an i.v. injection of chase hapten which forms a complex with the circulating antibody. The aggregates thus formed are removed rapidly by liver cells. Finally the radiolabeled antibody is injected i.v. and images taken (Goodwin D.A., *et al.*, 1986; 1987 and 1988).

6) Other approaches including the introduction of new bifunctional chelating agent without the problem of liver uptake and the modification of chelate-antibody binding site are being investigated to lower the liver uptake of radiolabeled antibodies (Brown B.A., *et al.*, 1986).

All of these modifications mentioned above help to promote the endeavor of controlling antibody background reduction. The techniques used to increase target to non-target ratio are as important as the improvements in antibody affinity, specificity and decreased antigenicity, to eventual successful clinical application of antibodies.

2.2. SQUAMOUS CELL CARCINOMA (SCC).

2.2.1. Etiology, Epidemiology and Management of SCC.

Squamous cell carcinoma (SCC) is the predominant histological type of tumor of the head and neck (Zarbo R.J. and Crissman J.D., 1988). The incidence of SCC of the head and neck accounted for 5-6% of all newly diagnosed malignancies in U.S.A and North-Western Europe (Schottenfeld D. and Bergard B.M., 1985; Blitzer P., 1988). This number is even larger in Mediterranean and South American countries where alcohol and tobacco consumption are higher (Killer A.Z.

and Terris M., 1965). This is because some of the causes attributed to the frequency of the SCC include higher alcohol and tobacco consumption (Killer A.Z. and Terris M., 1965) and human papilloma virus (Brandsma J.L. and Abramson A.L., 1989). The most frequent sites of metastasis of squamous cell carcinoma of the head and neck are lungs and cervix followed by the skeletal system (Snow G.B. *et al.*, 1986).

The traditional methods of clinical assessment of the status of patients with neck nodes, based on palpation, described in section 2.1.1. has a problem of producing high overall errors, 20-30%, due to false positive and false negative results (Ali S. *et al.*, 1985; Sako K. *et al.*, *1964*). Modern diagnostic methods like Ultrasound (U.S.), Computed tomography (C.T.) and Magnetic Resonance Imaging (M.R.I.), also described earlier in section 2.1.1.1., are not capable of detecting small localized nodes or their metastases. Clinically it is very important to eradicate the SCC node while it is still localized above the clavicles. In order to minimize metastasis the presence of the nodes have to be detected as early as possible. All of the methods known to date are not sensitive enough to detect very small nodes, therefore the need for a more sensitive diagnostic method became obvious.

A novel and promising approach which has the potential to solve this problem is the utilization of monoclonal antibodies (MAb) which are specifically immunoreactive with SCC cells. There have been many attempts by many researchers

to produce a monoclonal antibody which has a specific immunoreactivity with SCC tumor cells. The first monoclonal antibody produced against SCC cell surface antigen was by Zenner and Hermann. They documented that this MAb selectively killed laryngeal carcinoma cells, but they did not identify the antigen which was responsible for the initiation of the antibody (Zenner H.P. and Hermann I.W., 1981; Zenner H.P., 1985). Since then few monoclonal antibodies against SCC cells have been produced. Among them were three monoclonal antibodies, LuCa2, LuCa3 and LuCa4, produced by Kyoizumi S., *et al.*, against human SCC of the lung, but they were not tumor type specific and thus cross reacted with other tissue types (Kyoizumi S., *et al.*, 1985). Others like A9 and A10 MAbs, which were produced by Carry T.E., *et al.*, were directed against cell surface molecules of SCC (Carry T.E. *et al.*, 1983; Kimmel K.A. *et al.*, 1986; Kimmel K.A. and Carry T.E., 1986). Boeheim K., *et al.* produced SQM-1 antibody (IgM) which reacts preferentially with a 47 kd SCC surface antigen (Boeheim K., *et al.*, 1985). A MAb 17.13.C1.10, developed against a cytoplasmic antigen was found to be selectively expressed in squamous-cell epithelia and myoepithelial cells (Ranken R., *et al.*, 1987; Kaplan *M.J.*, *et al.*, 1987). Myoken Y., *et al.*, developed two murine monoclonal antibodies, B-10 and 1H5 against human oral SCC cells. While MAb 1H5 cross reacted with adenocarcinoma and with the spinous layer of normal stratified squamous epithelium, MAb B-10, reacted with non-epithelial and epithelial tissues and showed cross-

reactivity with other kinds of intermediate filament proteins (Myoken Y., *et al.*, 1987). Other research groups investigated the possibilities of utilizing MAbs produced against non-SCC tumors but found to be reactive with SCC of the head and neck (Yanagawa T., *et al.*, 1986; Hanzen-Logmans S.C., *et al.*, 1988). These antibodies are anti-CEA antibody, anti-epidermal growth factor (EGF) antibody and anti-MAM-6 antibody, all of which are not specific to SCC tumor and cross-react with other histological tumors and normal tissues (Cowley G., *et al.*, 1986). Quak J.J., *et al.* 1989, investigated three MAbs, E-48, K-984 and K-928, all of which were developed using SCC cells as an immunogen. Although all of them were immunoreactive with SCC tissues, E-48 cross reacted with adeno-carcinoma and large cell carcinoma, while the antigens which react with K-948 and K-928 were not identified (Quak J.J., 1989a, 1989b).

Most of the MAbs were produced utilizing the whole-SCC cell as the immunogen. As a result, antigens defined by these MAbs include cell surface proteins and cytoskeletal components of SCC cells (Myoken Y., *et al*, 1987; Fernstein F.D., *et al.*, 1986). Hence, none of the MAbs developed were sensitive and specific enough to be effectively used as a tool for the detection of SCC tumor. Recently MAb174H.64, which was developed against a non-SCC antigen, was reported to show selective reactivity with SCC tissue of different types (Samuel J., *et al.*, 1989). This report indicated that the antigen which is recognized by the MAb is a unique

epitope on cytoskeletal proteins. The objectives of this thesis include the identification, extraction and characterization of the SCC antigen which is recognized by MAb174H.64 and the investigation of the specificity of the immunoreactivity of MAb174H.64 with this antigen, as well as to observe the degree of localization of radiolabeled MAb174H.64 in SCC tumor in animal models.

2.2.2. Cytokeratin Proteins.

MAb174H.64 has been noted to selectively recognize antigens present in the basal cells of mammalian stratified squamous epithelium and squamous cell carcinoma. This antigen was suggested to be present in the cytokeratin protein extract of human and bovine SCC tissues (Samuel J., *et al.*, 1989). In order to isolate and characterize this antigen, the knowledge of its physical and chemical properties are crucial. A brief discussion on the composition of the SCC cytokeratin proteins is presented as following.

A large proportion of the cytoplasm of vertebrate cells, normal or transformed, is composed of cytoskeletal proteins, including actin-containing micro-filaments, tubulen-containing micro-tubules and intermediate filaments (I.F.) of 7-11 nm. There are five classes of I.F. proteins identified as specific to different tissue types. All of the I.F. proteins are morphologically identical in different cell types and are insoluble in solutions of a broad range of low or high salt concentrations and non-ionic detergents (Steinert P.M., *et al.*, 1981). However, on the bases of

immunological and biochemical criteria five different types of I.F. proteins have been identified (Bennett G.S., *et al.*, 1978; Frank W.W., *et al.*, 1978, 1981a; Hynes R.O. and Destree A.T., 1978; Lazarides E., 1980; Anderson B.H., 1981; Holtzer H., *et al.*, 1981; Osborn M., *et al.*, 1981). The five classes of I.F. proteins are: a) Cytokeratin filaments which occur in epithelial cells, b) Vimentin filaments which are characteristics of mesenchymally derived cells, serotoli cells, muscle cells and many cultured cell lines, c) Desmin filaments which are common in myogenic cells, d) Neurofilaments which are typical of neuronal cells, e) Glial filaments which are typical of astrocytes (Moll, R.W., 1982; Quinlan, R.A., *et al.*, 1986; Benz E.W., 1985). During cell transformation and tumor development, the cell type specificity of I.F. protein is largely conserved (Nelson W.G., *et al.*, 1984). Hence, it is possible to classify tumors histologically by identieying their specific type of intermediate filament (Schlegel R., *et al.*, 1980; Gabbiani G., *et al.*, 1981; Ramaekers F.C.S., *et al.*, 1981).

Squamous cell carcinoma tissues are epithelial types in origin. Hence, the intermediate filament proteins in SCC are cytokeratin polypeptides. The basic structure of cytokeratin proteins was suggested to be a dimer composed of type I (acidic) and type II (basic) keratins (Eckert R.L., 1989; Coulomb P.A. and Fuchs E, 1990). Each of the polypeptides contains two basic regions, a central α-helical rod domain with approximately 310 amino acids and amino- and carboxy-

terminal non-α-helical flanking domains (Stewart M., *et al.*, 1989; Steinert P.M. and Parry D.A.D., 1985; Quinlan R.A., *et al.*, 1986). The α-helical core seems to be of constant length and it is the amino- and carboxy-terminal regions of the different proteins that account for the variation in molecular weights of the different polypeptides of cytokeratins (Eckert R.L., 1989). In human and bovine epithelium and epithelial derived tissues, there are about 20 different cytokeratin polypeptides. Different epithelial cells contain different combination of the polypeptides ranging in molecular weight from 40 kd to 70 kd and isoelectric points from approximately pH 5.0 to 8.0 (Achtstaetter T., *et al.*, 1986; Doran T.I., *et al.*, 1980; Winter S., *et al.*, 1980; Fuchs E. and Green H., 1980, 1981; Franke W.W., *et al.*, 1981a, 1981b, 1981c; Milstone L.M. and McGuire J., 1981; Wu Y. and Rheinwald J.D., 1981). The acidic and basic polypeptides which paired to constitute the smallest unit of a cytokeratin protein usually differ in size by 8 kd, the basic being the heavier one (Benz E.W., 1985).

Although their unit structure is a dimer, cytokeratins exist in heterogeneous tetrameric complexes composed of two dimers (Aebi U., *et al.*, 1983). Cytokeratin proteins have a unique property which is not shared by the other I.F. proteins in that they are resistant to buffers containing non-denaturing detergents and high salt concentrations such as chlorides and iodides. A detailed description on the solubility and types of solubilizing agents used to

solubilize cytokeratin proteins is presented elsewhere (Achtstaetter T., *et al.*, 1986). Therefore, it is possible to characterize a given epithelium or epithelial cell by utilizing the specific pattern of its cytokeratin components.

2.3. MONOCLONAL ANTIBODIES.

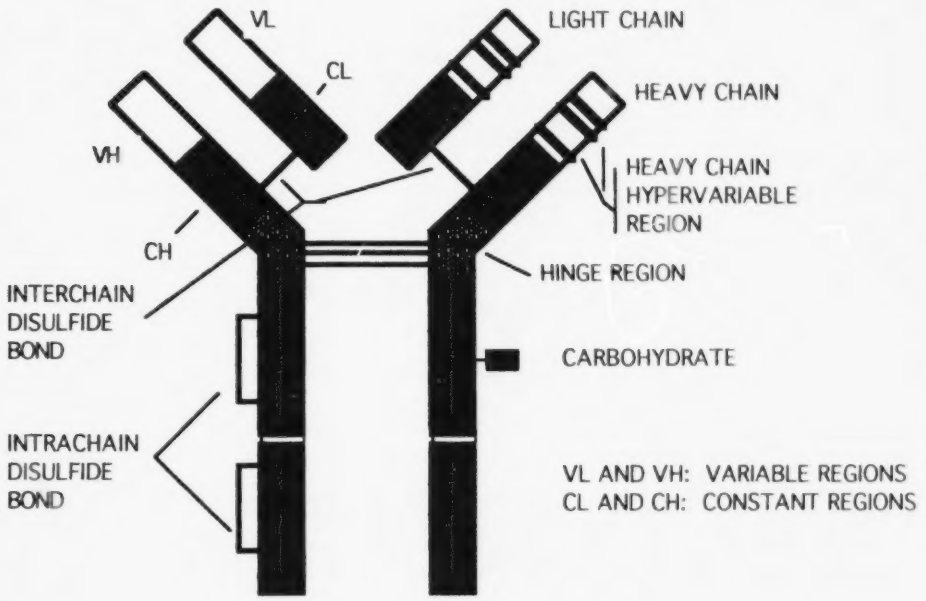
2.3.1. Introduction.

Protein molecules that combine specifically with antigens are termed antibodies. Since most foreign substances or antigens exhibit multiple antigenic determinants, the typical immune response is extremely heterogeneous. This is due to the different antigenic determinants which may stimulate the proliferation of multiple antibody-forming cell clones and the consequent secretion of heterogeneous antibodies. Each cell within a clone is secreting structurally identical antibody molecules. In 1975 Köhler G. and Milstein C. reported that it is possible to produce a cell line secreting antibodies which had a specificity for a pre-selected antigen. Detailed methods for monoclonal antibody production are available elsewhere (McCullough K.C. and Spier R.E., 1990 a, b and c). Briefly, antigen-stimulated spleen cells derived from a mouse that had been immunized with a known antigen, when fused with myeloma cells in poly-ethylene glycol (PEG), result in hybrid cells that can be cloned in a specialized medium which allows only the growth of the hybrid cell clones. These hybrid clones known better as hybridomas can be cloned and continuously cultivated either in tissue culture or mouse ascites

(Colnaghi M.I., 1991).

Five classes of antibodies have been identified in man and have been termed IgG, IgM, IgA, IgD and IgE. The first class, IgG antibodies, are usually the most important serum immunoglobulin in mammals and are currently thought to be the most useful for radioimmunoscintigraphy (RAIS) (Larson S.M., et al., 1985). A Schematic representation of an IgG molecule is presented in Figure 1. The main constituents are the two identical heavy chains and the two identical light chains. The four chains are held together by intra- and inter-disulfide bridges. Each of the heavy and light chains is further divided into a constant portion which is class specific and a variable portion which varies in amino acid sequence between various immunoglobulin molecules. Functionally the molecule is divided into two regions which include the antigen binding fraction F(ab) and the biological activity mediation fraction, which is also known as Fc fraction. The antigen binding fraction contains the hyper-variable regions responsible for the specific reaction of the antibody with an antigen. The Fc fraction has biological reactive regions such as complement binding regions and carbohydrate moieties.

Figure 1 Topology and functional architecture of the IgG molecule. (Adopted from Wasserman R.L. and Capra J.D., 1977)



2.3.2. Production and Purification of Monoclonal Antibodies.

2.3.2.1. Production of monoclonal antibodies

The advent of the development of hybridoma technology has made it possible for the generation of virtually unlimited quantities of monoclonal antibodies. The production of monoclonal antibodies in large quantities can be performed either in tissue cultures or ascites (Chandler J.P. 1987). Detailed descriptions of large scale preparation of

monoclonal antibodies are presented elsewhere (McCullough K.C. and Spier R.E., 1990a,b,c,d; Fazekas S., *et al.*, 1980). Briefly, production of monoclonal antibodies in large quantities can be done either in tissue culture or ascites fluid. The ascites method utilizes a mouse of the same genetic make-up as the hybridoma cell line and *in-vitro* intraperitoneal injection of the cell line. The cell line grows as an intraperitoneal tumor and the intraperitoneal fluid is collected from the intraperitoneal space. This fluid (ascites) is high in monoclonal antibody content. The yields of ascites fluid per mouse and monoclonal antibodies from different batches of mice injected with the same cell line were reported to vary within the range of 1.7 to 8.6 mL of ascitic fluid containing 2.9 to 17.3 mg/mL of monoclonal antibodies (Chandler J.P., 1987). The disadvantages of this method include; labor intensiveness and costly, variability in the quality and quantity of ascites the mice produce and the possibility of contamination with murine microbes. Hence, the use of animals for this type of work is now being discouraged (Chandler J.P., 1987).

The process of monoclonal antibody production through tissue culture is highly reproducible, large quantities (up to 3000 L) can be produced at one time, and the antibodies can be grown in serum-free media minimizing the need for intensive purification. Hence, it is the method of choice in bulk monoclonal antibody production. Tissue cultures can be at laboratory scale in roller bottles, in stirred vessels or

in small fermentors, or at industrial scales in either homogeneous culture suspension or immobilized or entrapped cell culture (McCullough K.C. and Spier R.E., 1990d).

2.3.2.2. Purification of monoclonal antibodies.

The development of monoclonal antibody technology and the need for high purity non-pyrogenic monoclonal antibodies required for *in-vivo* diagnostics (imaging) and immunotherapy, have created the need for new and more efficient purification methods. As a result, purification methods are constantly being scrutinized and optimized to achieve more homogeneous antibody preparations of acceptable quality. Most of the approaches to antibody purification currently in use are improved variations of the long established purification procedures used for serum polyclonal antibodies. Most of the commonly used antibody purification methods can be categorized according to one or more of the following mechanisms of separation. These are: precipitation, size exclusion, hydrophobicity, ion exchange and affinity. A brief communique of the purification method based on each of the above principles is presented here; detailed descriptions are available elsewhere (Parham P., *et al.* , 1982; Bruck C., *et al.*, 1986; Burchiel S.W., 1986; Juarez-Salinas H.J. *et al.*, 1986; Perkins A.C. and Pimm M.V., 1991a; Strickler M.P. and Gemski M.J., 1987; Nau D.R., 1987; Juarez-Salinas H.J., *et al.*, 1987).

One of the oldest and most useful methods of purification of immunoglobulins is based on the fact that

they are selectively precipitated by ammonium sulfate from serum (Parham P., *et al*, 1982). This precipitation method is gentle, effective and simple, but is not used as a final step of purification. It provides a substantial enrichment and reduces the protein load for subsequent purification steps.

The size exclusion chromatographic purification methods are based on the simple mechanism of sieving or molecular filtration by inert particles (pores) which serves as a stationary phase. The advantages of this method are its simplicity and the recovery of intact protein. However, this method is sensitive only for the separation of proteins with molecular weights differing by at least a factor of 2 (Regnier F.E., 1983).

The purification of monoclonal antibodies on the principle of hydrophobicity lies in the fact that all antibodies possess non-polar amino-acid residues which are hydrophobic. Antibodies are retained when allowed to pass through a stationary phase containing hydrophobic groups. An organic non-polar mobile phase is required to elute the antibodies. Since organic solvents are extremely denaturing to antibodies and the protein recovery is poor, this method needs more improvement before becoming useful (Hnatowich D.J., 1986)

Ion exchange chromatography is another important method for the purification of antibodies. This method is based on the mechanism of exchange of ions between the stationary phase and the mobile phase of a chromatographic system. All

proteins in solution are amphoteric and depending upon their isoelectric point (pI) and the pH of the solution, they can have either a net positive charge (pH < pI) or a negative charge (pH > pI) (Hnatowich D.J., 1986). At pH 8.0 almost all serum proteins will be negatively charged, and hence bind to an anion exchange matrix such as, diethylaminoethane (DEAE) which has a strong positive charge at this pH. Since immunoglobulins are the most basic of the major serum proteins they will be the first to be eluted. Traditionally, DEAE type anion exchange soft gel chromatography, with polysaccharides, dextran, cellulose, agarose, or polyacrylamides as support matrix, has been used for the purI.F.ication of monoclonal antibodies following ammonium sulfate precipitation (Bruck C., *et al.*, 1986). Although this method is simple and non-denaturing, it has some serious disadvantages which include, a) -its extremely low resolving ability between antibodies and the other serum proteins such as albumin and transferrin, b) -its low inherent capacity to bind proteins, c) -the short life of the column and d) -its tendency to bind immunoglobulin irreversibly which decreases antibody yield.

Hydroxylapatite is a non-specific immunoglobulin adsorbent which is very useful in the purification of monoclonal antibodies from contaminating mouse albumin, transferrin and other ascites proteins (Stanker L.H., *et al.*, 1985). Hydroxylapatite is an anion exchange type of chromatography which has hydroxylated calcium phosphate as

stationary phase (Hnatowich D.J., 1986). High Performance Hydroxylapatite (HPHT) has been found to be a useful tool to separate multiple idiotypes produced by certain hybridomas (Juarez-Salinas H., *et al.*, 1986).

The other very important method used for the purification of monoclonal antibodies is Affinity Chromatography. This method is based on the irreversible immobilization of one component of a system on a solid phase matrix and a subsequent binding and elution of a compound such as antibodies which have affinity to bind to the immobilized component (Porath J., 1974). This is an excellent one-step purification method. In Protein A affinity chromatography, Protein A, a cell wall protein from *Staphylococcus aureus*, is used as the immobilized component. It has a high affinity for the Fc portion of IgG antibodies (Goding W.J., 1978). Similarly, Protein G, a cell wall protein which is found in certain strains of *Staphylococcus* bacteria, also works in the same manner as Protein A affinity chromatography. Protein G differs from Protein A in that, while Protein G binds only to IgG antibodies, Protein A binds also with some IgM, IgA and IgE antibodies (McGuire J., 1989). In place of Protein A and G, a specific antibody or a specific antigen can be immobilized on the stationary phase and the system may be used for the isolation of specific immunogen or specific antibody respectively (Goding W.J., 1980).

2.3.3. Applications of Monoclonal Antibodies (MAbs).

Since the first MAbs were produced by Milstein and Kölher in 1975 (Kölher G. and Milstein C, 1975), numerous developments were made both in the improvement of hybridoma technology and the progress of refining antibody purification methods. This has facilitated the availability of many MAbs of high purity for use. At present there are many areas where the use of MAbs has become very important. Some of these include, applications in scientific research, diagnostic procedures, therapeutic uses and drug targeting. A brief account of each application is presented here and detailed information is available else where (McCullough K.C. and Spier R.E., 1990e).

The application of MAbs in scientific research fields contribute the largest portion of all of the MAb applications. Some of these areas are: 1) research in the studies of antigens and epitopes and their structures (Yewdall J.W. and Gerhard W., 1981; Yelton D.E. and Scharff M.D., 1981; Carter M.J. and Muelen V., 1984; Pollack R.R., *et al.*, 1984; McCullough K.C., 1986); 2) studies on the antigenic variation and epidemiology including viral, bacteriological and parasitological analysis of variations, and detections (Gerhard W. and Webster R.G., 1978; Yewdall J.W., *et al.*, 1979; Liver W.G., *et al.*, 1979); 3) as markers of immunologically important cells like leukocytes to understand the functions of the different lymphocytes (Möller G., 1982; Möller G., 1983; Baldwin C.L., *et al.*, 1986;

Gogolin-Ewin K.J., *et al.*, 1985; Puri N.K., *et al.*, 1985); 4) in cancer research MAbs have many applications including solid tumor detection, cancer therapy and analysis of tumor antigens (Yelton D.E. and Scharff M.D., 1981; Pressman D., 1980; Abdel-Nabi H.H., *et al.*, 1987). Although these represent the major applications of MAbs in scientific research there are other areas of research with some degree of applications (Vitetta E.S. and Uhr J.W., 1985).

The clinical application of MAbs in the diagnoses of diseases is based on its unique property to detect epitopes or changes on epitopes associated with the specific diseases (Liver W.G., *et al.*, 1979). The advent of the technology of radiolabeling of MAbs with the availability of different types of radioisotopes has expanded the applications of MAbs in the area of radio-immuno-scintigraphy (Perkins A.C. and Pimm M.V., 1991b). Table 1 summarizes some of the radiolabeled monoclonal antibodies used in the radioimmunodetection of human and animal cancers.

Drug targeting is one of the potentially very useful applications of MAbs. In order to get the required quantity of drug to reach the site of the pathogen or diseased tissue, MAbs can be used as carriers. Traditionally a high dose of drug is given to the patient, which in many cases, is not desirable because of the problem of drug toxicity. MAb specI.F.ic to an antigen associated to the pathogen or diseased tissue, can be conjugated to the drug and deliver it to the site, there-by reducing the dose used and avoiding the

Table 1 Radioimmunodetection of Diseases (cancers) using Radiolabeled Antibodies.

MAb	Antigen	Radioisotope	Disease (cancer) type detected	Reference
59DB	Fibrin	^{111}In	Thrombi	Knight L.C. et al, 1988.
7E3	11b/111a (Platelet)	$^{125}I, ^{111}In$	Venous Thrombi	Saito et al, 1988.
Z2D3	Ground part (Smooth muscle)	^{111}In	Atherosclerotic lesion	Kahw B.A. et al, 1992.
R11-D10	Myosin	^{111}In	Heart transplant rejection	Kahw B.A. et al, 1992.
103D2	126kd PGP[1]	^{111}In	Breast cancer (mammary)	Kim S.I. *et al*, 1988; Hnatowich D.J. *et al, 1992*; Khaw B.A. *et al*, 1986.

Continued.....

anti-sFv-RP-1	sFv-RP[2]-1	^{99m}Tc, ^{111}In	Myocardial Infarct	Nedelman M.A. *et al, 1993.*
anti-sFv	sFv[3]	^{99m}Tc, ^{111}In	Myocardial Infarct	Nedelman M.A. *et al, 1993.*
IMMU-4	CEA[4]	^{123}I	Colorectal carcinoma	Goldenberg D.M. *et al*, 1993.
B72.3	HMT[5]-TAG[6] 72	^{131}I	Colorectal carcinoma	Esteban J.M. et al., 1987.
250-30.6	HCC[7]-HT-29	^{131}I	Colorectal carcinoma	Leyden M.J. et al, 1986.
FO23C5	CEA[4]	^{131}I, ^{111}In	Colorectal carcinoma	Gasparini et al, 1988.
AUA1	PLAP[8]	^{125}I, ^{131}I	Colorectal Ovarian and breast carcinoma	Epenetos A.A. et al., 1986.
B14.011	CEA[4]	^{131}I	Colorectal carcinoma	Henze E. et al, 1987.
HMFG2	HMFGM[9]	^{123}I, ^{125}I ^{131}I	Ovarian carcinoma	Ward B.G. et al., 1987.

Continued.

PAY 276	HPAP[10]	^{111}In	Prostatic carcinoma	Babaian R.J. et al., 1987.
p96.5	HM[11]-P96.5	^{131}I, ^{111}In	Malignant melanoma	Murray J.L. et al., 1988.
225.28S	HM[11]-HMWA	^{123}I, ^{111}In ^{99m}Tc	Malignant melanoma	Carrasquillo et al., 1988.
9.2.27	HM[11]-HMW[12] cell surface antigen	^{111}In	Metastatic melanoma	Taylor A. et al., 1988.
ZME-018	MAA[13]-Gp 240	^{111}In	Metastatic melanoma	Kirkwood J.M. et al., 1987. Rainsbury R.M. et al., 1983.
3E1.2	HBC[14]	^{131}I, ^{111}In	Breast cancer	Esteban J.M. et al., 1987.
LGL-1D6	HG[15]	^{131}I	Frontal lobe glioma	Bergmann J.F. et al., 1987.
AF01	AFP[16]	^{131}I	Hepatocellular carcinoma	Bergmann J.F. et al., 1987.
T 101	CTCL[17]-T65 glycoprotein	^{131}I, ^{111}In	Cutaneous T-Cell Lymphoma	Keenan A.M. et al., 1987. Carrasquillo J.A. et al, 1987.

List of Abbreviations in Table 1.

1. PGP - Phosphoglycoprotein.
2. sFV-RP - Recombinant single chain Fv.
3. sFv - Single chain Fv.
4. CEA - Carcinoembrionic antigen.
5. HMT - Human mammary tumor.
6. TAG - Tumor associated antigen.
7. HCC - Human colorectal carcinoma.
8. PLAP - Placental alkaline phosphatase.
9. HMFGM - Human milk fat globulin membrane.
10. HM - Human melanoma.
11. HMWA - High molecular weight antigen.
12. MAA - Metastatic melanoma.
13. HBC - Human breast carcinoma.
14. HG - Human glioma.
15. AFP - Alphafetoprotein.
16. CTCL - Cutaneous T-cell lymphoma.

unnecessary exposure of normal tissues to the drug(McCullough K.C. and Spier R.E., 1990e).

Monoclonal antibodies are used in the passive immunization and therapy of viruses, bacteria and parasites (Carter M.J. and Meulen V., 1984; Pollack R.R., *et al.*, 1984). Based on the principle of drug delivery, MAbs, conjugated with cytotoxic drugs, have been used in the treatment of specific abnormalities (Vitetta E.S. and Uhr J.W., 1985).

2.4. THE ORIGIN OF MONOCLONAL ANTIBODY 174H.64.

2.4.1. Thomsen-Friedenreich Antigen.

The Thomsen-Friedenreich antigen (T-antigen) is a cryptic receptor on erythrocytes which is exposed when the red blood cell M N Glycoprotein is treated with neuraminidase (Springer G.F. and Desai P.R., 1975). The action of this enzyme is believed to remove the terminal sialic acid residues, thereby revealing the normal cryptic antigen, which is known as the Thomsen-Friedenreich antigen. T-antigen receptors are not accessible to anti-T antibodies because in healthy tissues they are shielded by O-glycosidically linked carbohydrates (Springer G.F., *et al.*, 1975). One of the possible reasons that tumor cells have their T-antigen receptors exposed could be due to the neuraminidase action of removing the shield masking the antigen (Springer G.F., *et al.*, 1979; Springer G.F., *et al.*, 1977).

2.4.2. Anti-Tn Antibody

When T-antigen is treated with an enzyme, ß-galactosidase, the Tn-antigen is recovered (Vainchenker W., *et al.*, 1985). Synthetic carbohydrate haptens, which are conjugated to carrier human serum albumin molecules [synthetic tumor-associated glycoconjugate (S-TAGs)] were used to immunize mice for the production of monoclonal antibody (Longenecker B.M. *et al.*, 1987). Utilizing this approach various monoclonal antibodies specific for Tn were generated.

Among the several MAbs prepared against the Tn antigen (S-TAGs), one showed immunoreactively with the Tn-HSA antigen. This antibody, MAb174H.64, On a random screening test exhibited a promising immunohistological staining pattern on squamous cell carcinoma (SCC) tissues. The clones were further recloned and the MAb174H.64 produced by the recloned cells continued to stain SCC but not the Tn-HSA any more. In normal human tissues this antibody was shown to react with the basal layer of the stratified squamous epithelia, with thymic epithelial cells and with myo-epithelial cells around the breast duct. Immuno-histopathology studies performed on patients' samples with different histological tumor types showed that MAb174H.64 reacts with SCC cells exclusively, but not with the other histological types. Similar results were reported for bovine, canine, feline and murine origin tissue types (Samuel J., *et al.*, 1989).

2.5. RADIOLABELING OF MONOCLONAL ANTIBODIES.

The increasing availability of specific monoclonal antibodies as targeting components and the possibility of attaching a suitable radioisotope has stimulated a relatively new field in nuclear medicine as diagnostic and therapeutic agents for cancer and related diseases. Monoclonal antibodies have been labeled with various types of radioisotopes for radioimmunodetection or radiotherapy of variety of tumor types (see Tables 2 and 3). Although there are a number of radioisotopes useful in the labeling of monoclonal antibodies as shown in Tables 2 and 3, the various methods of radiolabeling with the most important radio-nuclides such as ^{99m}Tc and radio-iodines are presented in the succeeding sections.

2.5.1. Labeling of MAbs with ^{99m}Tc.

^{99m}Tc is the preferred radio-isotope among the radionuclides currently used in diagnostic nuclear medicine. This is because its 140 keV principle gamma photon produces superior detection efficiency and image resolution with modern nuclear gamma cameras. Its 6-hr half-life and the lack of particulate radiation give low radiation exposure to the patients. The other advantages of this radioisotope are its ease of availability from a generator and its relatively low cost.

Table 2. Selected radioisotopes used for radioimaging*.

Radioisotope	Half-Life	Major Decay Mode	Major γ Energy keV, (%)
^{99m}Tc	6.0 hr	I.T[1].	140, (90)
^{123}I	13.3 hr	E.C[2].	159, (83.2)
^{131}I	8.0 d	Beta	364, (81.2)
^{67}Ga	3.3 d	E.C[2].	93, (37)
			185, (20.4)
			300, (16.2)
^{68}Ga	68.3 m	Positron	511, (176)
		E.C[2]	1080, (3.5)
^{111}In	2.8 d	E.C[2].	172, (91)
			247, (94.2)
^{113m}In	99.5 m	I.T[1].	392, (64.9)

*Ledrer M. and Shirley V.S.,(eds) "Tables of Isotope" 7th Ed., Wiley, New York,1978 and from Radiopharmaceuticals, G. Subramanian *et al* (eds.), The Society of Nuclear Medicine Inc., New York, 1975.

[1] I.T. = Isomeric Transition.

[2] E.C. = Electron Capture.

Table 3. Radioisotopes used for monoclonal antibody therapy.*

Radioisotope	Half-Life	Decay Mode	Particulate Maximum Energy(MeV)
Astatine-211	7.0 hr	Alpha	5.9
Iodine-131	8.0 d	Beta	0.6
Rhenium-186	3.5 d	Beta	1.1
Rhenium-188	17 hr	Beta	2.1
Yttrium-90	2.5 d	Beta	2.3
Iodine-125	60 d	E.C.[1] (Auger)	0.035
Copper-67	62 hr	Beta	0.18
Bismuth-217	1 hr	Beta	8.78
Phosphorus-32	14.3 d	Beta	1.70
Scandium-47	3.4 d	Beta	0.16

*From 'Monoclonal Antibodies in The Diagnosis and Treatment of Cancer' 2nd edition, Foon K.A., 1989, ed. NeoRx Co. and Mallinckrodt Inc.

[1] E.C.= Electron Capture.

There are three major approaches in the process of labeling monoclonal antibodies with technetium. These are direct labeling of the antibody, indirect labeling through chelating agents attached to the antibody and thirdly indirect labeling using prelabeled chelate (Eckelman W.C. and Steigman J., 1991, 1989; Hnatowich D.J., 1990b).

2.5.1.1. The direct labeling method.

The direct labeling of proteins with ^{99m}Tc has been the subject of recent reviews by Rhodes B.A., 1991; Eckelman W.C. and Steigman J., 1991; Hnatowich D.J., 1990 a,b and Delmon-Moingeon L.I., *et al.*, 1991. A brief description of the principles of this labeling method is presented here. Antibodies contain a number of naturally present functional groups such as $-NH_2$, -OH, -COOH or -SH. As a result, the direct labeling of antibody with ^{99m}Tc can involve two binding sites: low affinity, high capacity binding sites, related to free $-NH_2$, -OH and -COOH groups, and high affinity, low capacity binding sites related to sulfhydryl (-SH) groups. Since the -SH groups provide the high affinity binding site resulting in a strong stable bond with ^{99m}Tc, it is preferred to the low affinity binding sites which are unstable. Therefore only the direct labeling method of ^{99m}Tc to antibodies, which is based on the mechanism of bonding of ^{99m}Tc with reactive sulfides ($-S^-$) or sulfhydryl group present in antibody molecules, will be discussed. Antibodies have an abundant quantity of cysteine residues linked to each other, forming either intra- or inter-chain disulfide bridges. There

are two ways by which a protein molecule can be treated to generate a sulfhydryl group or a reactive sulfide moiety. The first is to reduce native disulfide bridges by incubating the protein with reducing agents such as stannous ions (Rhodes B.A., *et al.*, 1980; Rhodes B.A., *et al., 1986;* Pettit W.M., *et al.*, 1980) or other reducing agents such as ascorbic acid, dithiothreitol (DTT), 2-mercaptoethanol, ferrous ions (Thakur M.L., *et al.*, 1991; Schwarz A., *et al.*, 1987; Lin M.S., *et al.*, 1971). A report on the comparison of these reducing agents suggested ascorbic acid to be a better reducing agent for disulfide bonds (Thakur M.L., 1990). However, the preservative effect of stannous ion on the reduced sulfhydryl moieties is an extra advantage. The second way is to introduce a free sulfhydryl moiety by incorporating -SH containing bifuctional chaletes such as 2-iminothiolane, in to the protein (Joiris E., *et al.*, 1991).

The mechanism of direct labeling of antibodies with ^{99m}Tc requires not only the reduction of the disulfide group to the sulfhydryl or reactive sulfide group, but also the reduction of the ^{99m}Tc to the required redox state. In order to protect the reduced moieties from oxidation or unwanted reaction they have to be preserved with complexing agents. Generally, the labeling of antibodies with ^{99m}Tc via the $SnCl_2$ reduction method can be summarized in three main steps, as shown in Figure 2, and these are:

(1) The reduction of disulfide groups in the antibody with out causing damage to the immunologically reactive sites

and the protection of the reactive sulfide moiety by stabilizers such as stannous or zinc ions (Eckelman W.C. and Steigman J., 1991) or protecting the free -SH groups by derivatizing them with a reagent that can be subsequently removed (Stark G.R., 1964).

(2) The reduction of ^{99m}Tc ion to a redox state reactive with sulfide moieties and the subsequent preservation of the reduced ^{99m}Tc by weak chelating agents such as tartarate and glucoheptonate.

(3) Finally, the interaction between the reduced ^{99m}Tc and the reactive sulfide group to give ^{99m}Tc labeled antibody

Figure 2 Reactions in the direct method of labeling antibodies with ^{99m}Tc (Rhodes B.A., 1991).

1. $R\text{-}S\text{-}S\text{-}R + SnX_2 \longrightarrow 2\ R\text{-}S\text{-}Sn\text{-}X$

2. $TcO_4^- + SnX_2 + \text{tartarate} \longrightarrow Tc\ (\text{tartarate})$

3. $Tc\ (\text{tartarate}) + R\text{-}S\text{-}Sn\text{-}X \longrightarrow R\text{-}S\text{-}Tc\ X_2$

List of Abbreviations

R-S-S-R = antibody;

SnX_2 = stannous ion;

R-S-SnX = reactive sulfide moiety protected by stannous ion;

Tc (tartarate) = Tc preserved by the weak chelating agent;

$R\text{-}S\text{-}TcX_2$ = Tc labeled antibody.

(Rhodes B.A., 1991). The various alternative methods of antibody reductions are summarized in a review article by Rhodes B.A., 1991.

Although the direct labeling approach is simple and provides a one step labeling method, it has some disadvantages. These include, the uncertainty of the labeling site which can occur at any of the low or high affinity sites resulting in variable stability (Delmon-Moingeon L.I., *et al.*, 1991) and the formation of tin-^{99m}Tc colloid when stannous chloride is used as a reductant (Pettit W.M., *et al.*, 1980). Some of the problems encountered in the direct labeling method have been improved by employing several 1991. alterations in the method of labeling. The most important ones are:

(1) Increase in the number of the high affinity binding sites i.e. sulfhydryl group (-SH); the -SH moieties have been shown to increase by incubating the antibody with the stannous ion for a prolonged period of time or by utilizing the pretinning method (Rhodes B.A., *et al.*, 1986).

(2) Decreasing the low affinity sites; DTPA binds to the low affinity sites. Therefore labeling antibodies with ^{99m}Tc in the presence of DTPA prevents the labeling of low affinity sites without affecting the binding processes at the high affinity sites (Paik C.H., *et al., 1985*).

(3) Minimizing tin-^{99m}Tc-colloid formation. When stannous ion is used as reductant, the solution of the preparation can be made acidic with in a pH range of 3.1-3.2 to avoid colloid

formation (Pettit W.M., *et al.*, 1980).

(4) Other ways of preventing formation of radiocolloid include the use of sodium azide in concentrated hydrochloric acid to reduce the ^{99m}Tc to a nitrido-reactive intermediate, $^{99m}TcNCl_4^-$ (Kanellos J., *et al.*, 1986). In this approach the antibody is partially reduced to convert disulfide bonds into sulfhydryl residues, using DTT as a reducing agent. The reaction between the $^{99m}TcNCl_4^-$ and the sulfhydryl residue is very rapid and the complex formed is very stable with no colloid present (Baldas J., *et al.*, 1985). Similarly a technetium nitride intermediate species produced by heating a mixture of N,N, dimethylformamide, HCl and ^{99m}Tc pertechnetate in normal saline has been shown to efficiently label antibodies without colloid formation (Feitsma R.I.J., *et al.*, 1987; Bolk D., *et al.*, 1989). The reactive-nitrido prepared in the latter way reacts with the amino groups of the lysine residues of the protein, hence this process does not require the reduction of the disulfide groups of antibodies and therefore there is minimal problem of Sn-colloid formation.

2.5.1.2. The indirect labeling method.

The indirect labeling method is based on the principle of labeling antibodies with ^{99m}Tc through chelate bridges. In this type of labeling method, strong bifunctional chelating agents (BFC) are used to bind antibodies and reduced ^{99m}Tc. The chelate covalently attaches itself to the antibody at the amide ($-NH_2$), sulfhydryl group (-SH) or to the -OH of

carbohydrate moiety and to the reduced ^{99m}Tc on its other functional moiety, producing technetium labeled antibody through a chelate bridge (Yuanfang L. and Chuanchu W, 1991; Hnatowich D.J, 1993). There are a variety of methods employed in the technique of labeling antibodies with reduced ^{99m}Tc through chelate bridges. Most of these variations depend upon the bifunctional chelates used, the site where the chelate attaches to the antibody and whether the chelating agent is attached to the reduced ^{99m}Tc before or after the coupling process with the antibody is completed.

There are a number of chelating agents used in the labeling of antibodies with ^{99m}Tc. A list of bifuctional chemical chelating agents which belong to the polyaminopolycarboxilic acid group is presented with their chemical structures in Figure 3. The first bifunctional chelating agent, developed by M.M. Sundberg, *et al.*, 1974 was a polyaminopolycarboxylic acid, ethylenediaminetetraacetic acid (EDTA) and its derivatives. One of them, 1-(p-aminophenyl)ethylenediaminetetraacetic acid, when diazotized and mixed with antibodies, attaches mainly to the tyrosine, histidine and lysine residues by diazonium coupling (Meares C.F. and Goodwin D.A., 1984). Other analogs of EDTA are also described elsewhere (DeRiemer L.H., *et al.*, 1979; Yeh S.M. *et al.*, 1979; Leung C.S.H., *et al.*, 1979; DeRiemer L.H., *et al.*, 1981; Meares C.F., *et al.*, 1984; Meares C.F. and Wensen T.G., 1984).

Diethylenetriaminepentaacetic acid (DTPA) and its derivatives represent a group of chelating agents with an extra aminocarboxylic acid group and are found to be superior to EDTA with regard to chelating potential (Lanteigne G. and Hnatowich D.J., 1984). The most popular DTPA derivatives are mixed anhydride (Khaw B.A., *et al.*, 1982) and cyclic anhydride (Paik C.H., *et al.*, 1983a) and their derivatives. The latter is more stable to hydrolysis and is preferred to the mixed anhydride, therefore the proteins may be coupled with reproducible efficiencies (Hnatowich D.J., *et al.*, 1983). However, the cyclic anhydride has a disadvantage in that it may form dimers by cross linking two antibodies through a single DTPA group (Hnatowich D.J., 1989a; Hnatowich D.J., *et al.*, 1983; Zoghbi S.S., *et al.*, 1985). Other derivatives of DTPA like N-hydroxy succinimide ester have been reported to be more stable than bicyclic anhydrides of DTPA. The coupling efficiency of the DTPA pentaester has been found to be superior to that of bicyclic anhydride DTPA (Najafi A., *et al.*, 1984; 1986). However the label formed by this method is found to be less stable *in-vitro* and *in-vivo*. The N-hydroxysuccinamide monoester of DTPA has been reported to have the advantages of preventing the intra- and inter-molecular coupling of antibodies (Paxton R.J., *et al.*, 1985; Jakowatz J.G., *et al.*, 1985). A synthetic DTPA derivative, 1-(p-isothiocyanatobenzyl) DTPA has one more carboxylate group to bind to the metal ion than does any one of the DTPA derivatives mentioned above (Brechbiel M.W., et al., 1986;

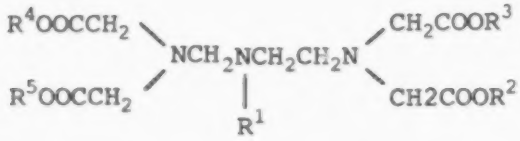
Esteban J.M., *et al., 1987*; Paik C.H., *et al.*, 1983a.).

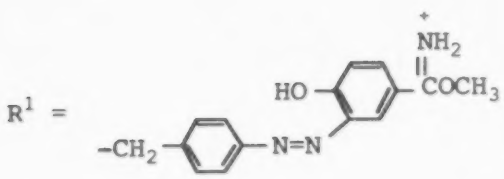
Another polyaminopolycarboxylic acid used as a bifuctional chelating agent is triethylenetetraaminehexa-acetic acid (TTHA). It has six carboxyl groups available for coupling with proteins and metal ions and therefore it is expected to have stronger chelation ability than DTPA because of the extra carboxylic group. However, a report showed no significant difference in their organ localization in an animal model (Buckley R.G., *et al.*, 1986). Similar derivatives, such as N-hydroxysuccinimide monoester, prepared from DTPA are also prepared for TTHA (Buckley R.G., *et al.*, 1986) and bicyclic anhydride (Hnatowich D.J. and McGann J., 1987).

The coupling of protein with polyaminopolycarboxylic acids such as DTPA involves the conjugation of the acyl carboxylic acid groups with the C-amino group of the lysine residues of antibodies. The DTPA-antibody complex, once formed and purified, is mixed with stannous ion reduced ^{99m}Tc or by transmetallation of ^{99m}Tc glucoheptonate, to produce ^{99m}Tc labeled DTPA antibody complex (Delmon-Moingeon L.I., *et al.*, 1991). The disadvantages of this method include: (1) non-specific binding of ^{99m}Tc to the protein resulting in weak unstable bonding (Lanteigne D., *et al.*, 1984). (2) The number of DTPA chelates per antibody molecule could affect the immunoreactivity of the antibody by creating steric hindrance (Paik C.H., *et al.*, 1983b).

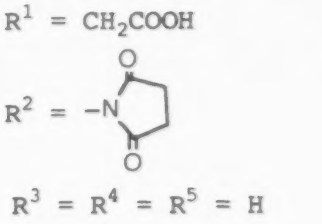
Figure 3 The chemical structure of polyaminopoly-carboxylic acids used as chelating agents.(Adopted from Yuanfang L. and Chuanchu W., 1991)

A. Derivatives of diethylenetriaminepentaacetic acid (DTPA)

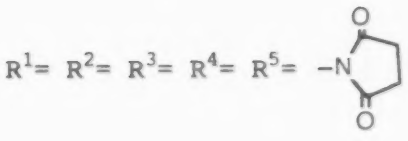




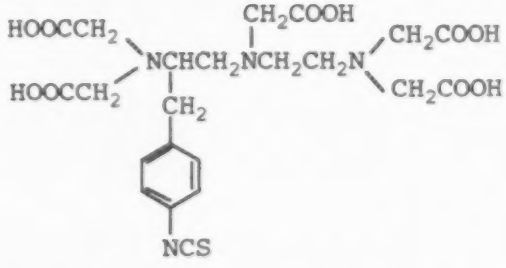
DTTA azo imidate



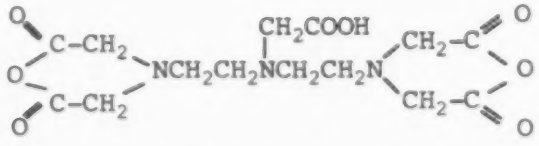
N-hydroxysccinimide monoester of DTPA



N- hydroxysccinimide pentaester of DTPA



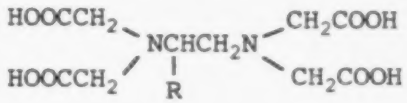
1-(p-isothiocyanatobenzyl) DTPA

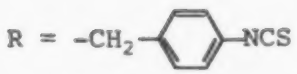


Bicyclic anhydride of DTPA

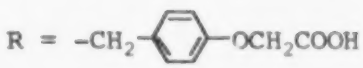
Figure 3 (continue)

B. Derivatives of ethylenediaminetetraacetic acid (EDTA).

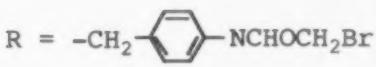




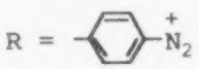
1-(p-isothiocyanatobenzyl)
EDTA



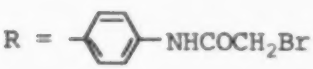
1-(p-carboxymethoxybenzyl)
EDTA



1-(p-brromoacetamidobenzyle)
EDTA

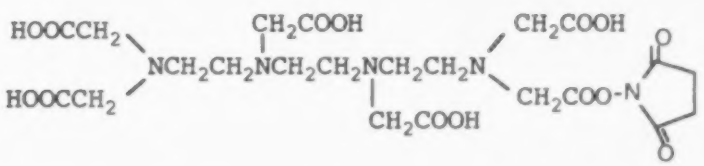


1-(p-benzendiazonium)
EDTA

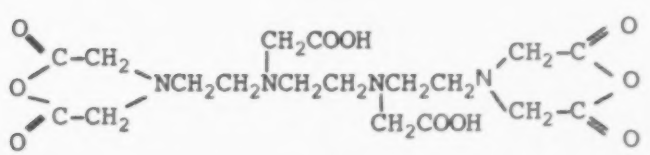


1-(p-bromoacetamidophenyl)
EDTA

C. Derivatives of triethylenetetraaminehexaacetic acid (TTHT)



N-hydroxysuccinimide monoester
of TTHA



Bicyclic anhydride
of TTHA

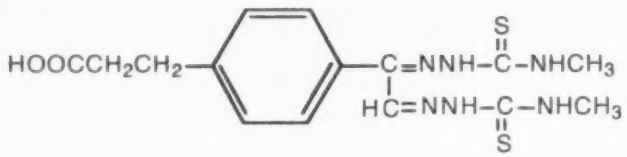
Sulfur containing ligands are the other class of bifuctional chelating agents different from the polyaminopolycarboxylic acid groups of BFC. The chemical structures of bifunctional chelating agents containing sulfur atoms is presented in Figure 4. There are three sub-classes in this group, differing in their chemical structures: 1) Bifuctional chelating agents containing bis-thiosemicarbazone groups such as p-carboxyethylphenylglyoxal-di-(N-methylthiosemicarbazone) (CE-DTS), have shown high *in-vitro* and *in-vivo* stability (Arano Y., *et al.*, 1987). 2) Diamidodimercaptide ligands (N_2S_2) including diaminodithiol and diaminodithiolthiolactone have been shown to form stable complexes with ^{99m}Tc (Liang F.H., *et al.*, 1987; Efange S.M.N., *et al.*, 1988; Fritzberg A.R., *et al.*, 1986; Kasina S., *et al.*, 1986). Costello C.E. *et al.* synthesized about twenty diamidodimercaptide (N_2S_2) derivatives to produce ^{99m}Tc complexes of desirable structures and high stability (Costello C.E. *et al*, 1983; Fritzberg A.R. *et al*, 1986). It has been reported that two approaches to ^{99m}Tc antibody labeling with the N_2S_2 ligand are possible. The first one is the conjugation of ligand to antibody and then exchange of the metal ion from a weak complex to the N_2S_2-antibody conjugate (Hwang K.M. *et al*, 1986). This procedure requires a more rigorous reaction condition including pH 8 and 50^{o}C both of which are likely to affect the integrity of the antibody. An alternative approach is the "pre-formed chelate approach" method which is the labeling of the chelate with ^{99m}Tc prior

to the conjugation to the antibody (Eary J.F., *et al.*, 1989). Other bifuctional chelating agents have been described recently which employ the pre-formed chelate approach. These include SCN-derivatives of aromatic boronic acid reagents (Linder K.E., *et al.*, 1990) which are bifuctional chelating agents for proteins and preformed chloro-trisdimethylglyoxime technetate [$TcCl(DMG)_3$] complexes. Derivatives of 1,4,8,11-tetra-azacyclotetradecane (cyclam) such as aminopropyl methylcyclam (MAC) first form a very stable Tc-MAC complex and then react with the antibody, attaching strictly to the sulfide moiety of the antibody (Franz J. *et al*, 1987). The structures of cyclam derivatives are presented in Figure 5.

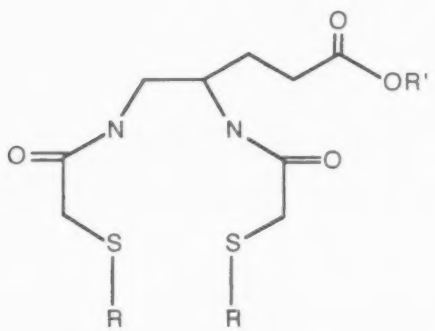
The immunoreactivity of antibodies can be affected by covalent attachment of BFC closer to the immunosensitive antigen binding sites of the immunoglobulin. One radical approach to improve this situation is to find a way to attach chelates to the carbohydrate moieties of antibodies which are strictly localized in the constant domain of the immunoglobulins (Brown B.A., *et al.*, 1986; Rowell J.D., *et al.*, 1986). It was suggested that oxidation of the oligosaccharide residue of the antibody to an aldehyde *al.*, 1991). This approach has the advantage over the previously discussed ones, in that the chelation and labeling sites are further away from the immunologically sensitive site. The high liver, kidney and spleen uptakes observed whenfunctional group provides a reactive site for bifuctional compounds

Figure 4 The chemical structures of bifunctional chelating agents containing sulfur atoms (Adopted from Yuanfang L. and Chuanchu W., 1991).

A. p-Carboxyethylphenyl di(N-methylthiosemicarbazone) (CE-DTS).

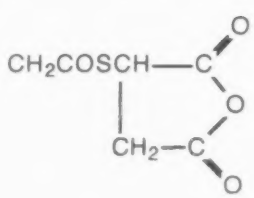


B. Diamide dimercaptide ligands.



R = thiol ester R' = active ester

C. Acetylthiosuccinic anhydride.



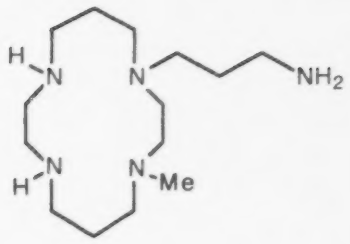
which contain amine, hydrazine, hydrazyde and semicarbazide groups (Delmon-Moingeon L.I., *et* antibody conjugated to DTPA by conventional method is used was reported to be minimized when the same study was done using the activated oligosaccharide sites for conjugation with DTPA (Ultee M.E., *et al.*, 1989).

Another improvement made in the antibody-chelate labeling is directed to reducing radioactivity in the blood and minimizing liver retention of the radioactivity. This approach is based on the use of linker groups between the antibody and the chelate which when metabolized allow the radiolabeled metabolic product to be easily cleared from the system. The linker group must be easily cleavable/ hydrolyzable. Metabolizable linkers actively or potentially useful are esters, diesters and disulfide groups (Meares C.F., *et al.*, 1988; Deshpande S.V., *et al.*, 1989; Quadri S.M., *et al.*, 1986; Paik C.H., *et al.*, 1989).

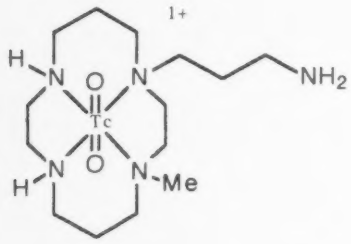
2.5.2. Radioiodination.

Radioisotopes of iodine are the longest standing and most utilized radioisotopes still in use. Among the many radioactive isotopes of iodine, there are three which are commonly used in different research disciplines and clinical practices. These are ^{131}I, ^{125}I and ^{123}I. The major decay modes and the half lives for ^{131}I, ^{125}I and ^{123}I are presented in Figure 6.

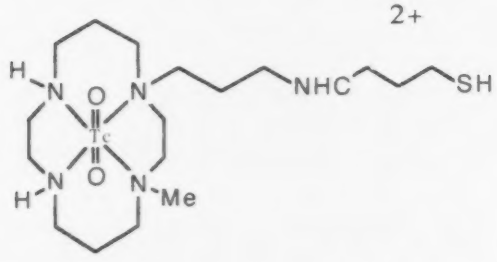
Figure 5 Structures of Cyclam derivatives.(Adopted from Delmon-Moingeon L.I., et al., 1991)



1-(3-aminopropyl)-4-methyl-1,4,8,11-tetraazacyclotetradecane (MAC)



Tc-MAC



Tc-MAC-SH

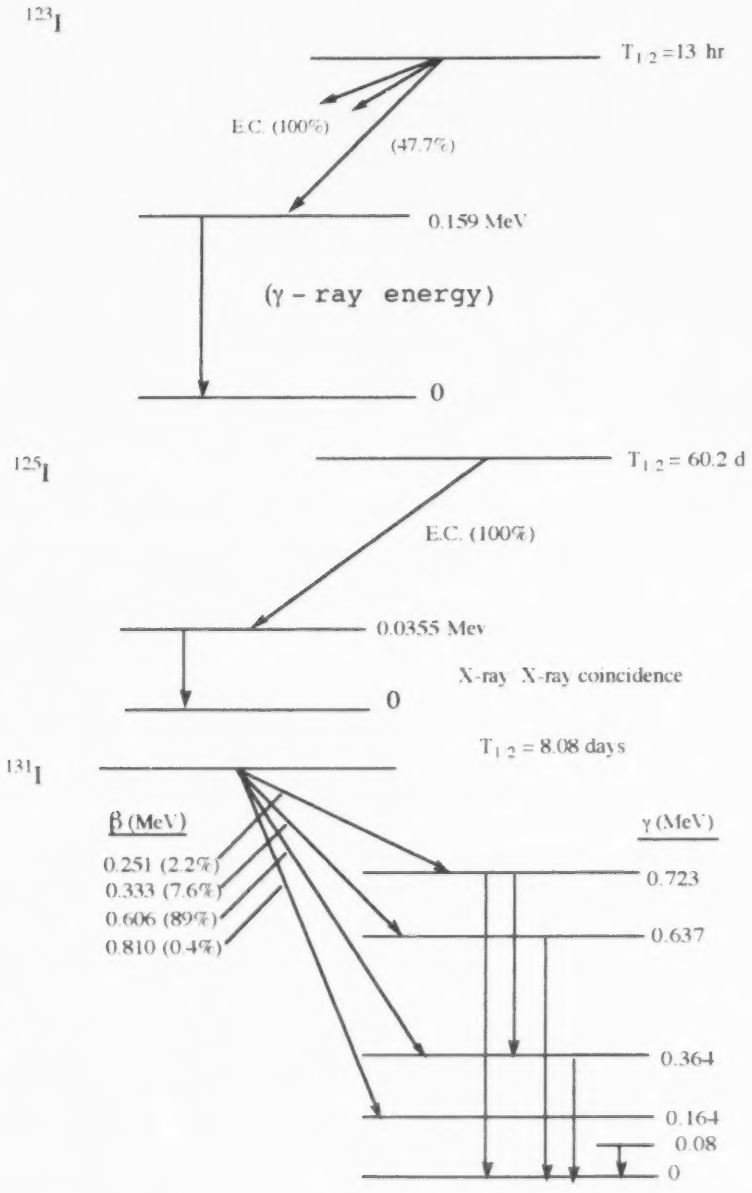
Iodine [^{131}I] has a principle gamma energy of 364 keV which is considered to be higher than ideal for scintigraphy. Its long half-life, 8 days, in association with the beta emission contributes to unnecessary radiation dose to patients. Inspite of this ^{131}I is currently very popular in the diagnoses and therapy of thyroid diseases.

Iodine [^{125}I] with its very low principal gamma energy cannot be used for *in vivo* imaging studies, as the 35 keV photon is significantly attenuated by overlying tissues. However, the low energy photon emission permits enhanced counting efficiency, which along with the 60 days half-life has made this radioiodine very useful for *in-vitro* studies.

Iodine [^{123}I] has very superior physical characteristics ideal for imaging purposes (Epenetos A.A., *et al.*, 1985; Delaloye B., *et al.*, 1986), but its high cost and relatively poor availability has limited its usefulness. For scintigraphy studies involving radiolabeled monoclonal antibodies, the maximum uptake may require 2-3 days following administration. Since the physical half-life of ^{123}I is short for the procedure, high activities of the labeled monoclonal antibodies might be required.

The methods of incorporation of the iodine atom into protein have been the subject of extensive reviews (Baldwin, R.M. and Lin T.H., 1982; Bolton, A.E., 1977). The first step in the radioiodination procedure involves the oxidation of the radioiodine in the presence of the protein or other molecules containing tyrosine residues. Iodous ion (I^+)

Figure 6 Major decay modes and half-life for a) ^{123}I, b) ^{125}I and c) ^{131}I radioisotopes (Adopted from Horrocks D.L., 1981).



(Bolton, A.E., 1977) does not exist as such, but usually forms the reactive species $[H_2OI]^+$, known as hydrated iodous ion with a nucleophilic species in aqueous solution (Dewanjee M.K. and Rao S.A., 1983).

Although the tyrosine residue provides the primary and the best site for the iodination of proteins, there are other secondary sites which can be radioiodinated, such as the histidine residue, (Li C.H., 1944) and the cysteine residue (Hughes W., 1957). The secondary protein binding sites undergo iodination at a much slower rate than tyrosine and are not as stable. Therefore the tyrosine residue is considered to be the best site for the radioiodination of proteins.

The basic principle in the process of radioiodination is the introduction of an electrophilic species "I^+" (iodous ion) either directly into the ortho position on the phenol ring of tyrosine residue of the protein or indirectly by first introducing the iodous ion into a tyrosine moiety of a chelating agent and later attaching the radioiodinated chelate to the protein (Hnatowich D.J., 1989b; Bolton A.E., 1977). In both approaches the presence of the iodous ion is vital. The introduction of the iodous ion into the tyrosine residue can be achieved by one of the two reaction path ways presented in Figure 7. These are: substitution reaction, where iodous ion replaces a hydrogen atom of the tyrosine residue or by isotope exchange, were the radioactive iodous ion is exchanged with a stable iodine atom already attached

to the tyrosine residue.

There are at least five methods known for the oxidation of radioiodines into the reactive iodous ion for the process of direct labeling proteins. These are briefly presented in the following section under direct iodination method.

Figure 7 Mechanisms of radioiodination of proteins at the tyrosine residue (Adopted from Markwell M.K., 1988).

A) Substitution reaction:

$R\text{-}H + {}^{*}I_2 \quad <=======> \quad R\text{-}{}^{*}I + HI$

B) Isotope exchange:

$R\text{-}I + {}^{*}I_2 \quad <=======> \quad R\text{-}{}^{*}I + I_2$

${}^{*}I_2$ = Radioiodine

R-H = Protein

R-*I = Radioiodinated protein.

2.5.2.1. Direct iodination methods.

A) The molecular iodine (I_2) and the iodine monochloride (ICl) methods .

These two approaches were the earliest methods in the process of labeling proteins, but since then are being replaced by more efficient procedures, they are now only of historical interest (Hnatowich D.J., 1989b). In these methods either ICl or I_2 reacts with sodium radioiodide, Na*I, to form *ICl or *I-I respectively. In the presence of glycine buffer, both *ICl and *I-I produce hypoiodate which labels the protein. The reaction can be terminated by adding sodium metabisulfite. The advantages of these methods include the simplicity and availability of the reagents used. The disadvantages are the incorporation of non-radioactive iodine into the protein, which decreases the specific radioactivity of the labeled protein, and the slow rate of reaction, that results in exposure of the protein to damaging radiation (Yalow R.S. and Berson S.A., 1960; McFarlane A.S., 1958) .

B) The chloramine-T method.

Chloramine-T is the sodium salt of N-chloro-p-toluene sulfonamide. In aqueous solution it slowly produces hypochlorous acid which is a mild oxidizing agent (Bolton A.E., 1977). For radioiodination of antibodies by this method, the protein, in buffered solution (pH 7-8), is allowed to mix with the radioiodine followed by addition of an aqueous solution of chloramine-T (Hunter W.M. and

Greenwood F.C., 1962). The reaction times vary but are usually in the range of two to five minutes (Perkins A.C. and Pimm M.V., 1991c). The reaction is terminated by the addition of an appropriate amount of reducing agent such as sodium metabisulphite. The advantages of this method are the ease and the speed at which the reaction occurs and the high degree of iodine incorporation and the high specific activity of the labeled protein. Excessive exposure of proteins to Chloramine-T causes damage to the immunologically reactive sites (Bolton A.E., 1977).

The chloramine-T method is widely used for the radioiodination of antibodies, hormones and other proteins. This procedure uses relatively large quantities of reducing agent to terminate the oxidation process as well as to counteract the excess oxidizing agent present, at the end of the labeling process. In order to improve this condition dichloramine-T a water-insoluble derivative of chloramine-T has been reported for radioiodination in non-aqueous media (Jyotsna T., *et al.*, 1986). The dichloramine-T is dissolved in chloroform and transferred into a clean glass reaction vial and dried under a stream of nitrogen. Protein in phosphate buffer at pH 6.0 mixed with the appropriate amount of radioiodine is added into the reaction vial. The reaction is terminated when the solution is removed from the reaction vial. Therefore, in this case there is no need for reducing agent and the problem caused as a result of excess reducing agent is avoided.

C) The electrolytic method.

This method utilizes a constant current electrolysis to convert iodide to iodine instead of using chemical oxidation method to do the same. This is an efficient and mild procedure for the iodination of proteins (Pennisi F. and Rosa U., 1969). In this method proteins are not exposed to either oxidizing or reducing agents, therefore, the resulting product is stable, has a high specific activity and retains high levels of immunological and biological activities. The disadvantages of this method are that the proteins are exposed to the potentially damaging radioiodine solution and that it is kept diluted at room temperature for a long time, conditions which are considered to be damaging to some proteins. This method requires specialized equipment and the technique is more complex to perform than the simple chemical iodination.

D) The enzymatic iodination.

Enzymes like lactoperoxidase are used to catalyze the oxidation of iodide in the presence of a small amount of hydrogen peroxide (Marchalonis J.J., 1969; Morrison M. and Bayse G.S., 1970). Protein, radioiodine and lactoperoxidase are first mixed in a reaction vessel, then hydrogen peroxide is added to start the labeling reaction. The labeling reaction is maintained by adding hydrogen peroxide every 10 minutes. The reaction is terminated by the addition of cysteine (Miyachi Y., *et al.*, 1972). The quantity of the reagents, the frequency of addition of the hydrogen peroxide

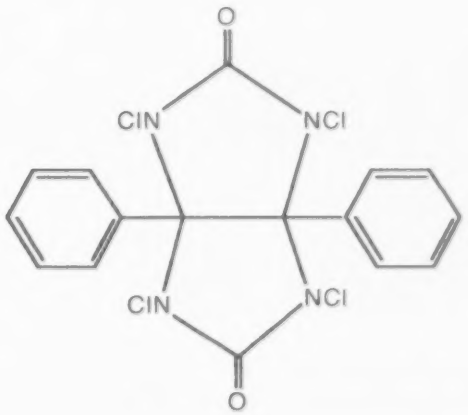
and the time of incubation affect the yield of the iodination reaction. The optimum conditions vary from one type of protein to another (Miyachi Y., *et al.*, 1972). This method minimizes labeled protein damage as it avoids exposure of the protein to oxidizing or reducing agents (Miyachi Y. and Chrambach A., 1972) and maintains the integrity of the immunological and biological activities (Miyachi Y.. *et al.*, 1972; Krohn K.A. and Welch M.J., 1979). In addition to its use for radioiodinating tyrosine residue, this method has been used to label the histidine residues of protein (Krohn K.A. and Welch M.J., 1979). The disadvantages of this method are low yield of iodination and low specific activity, as well as the introduction of impurities which come from the labeling of the lactoperoxidase itself.

E) The Iodogen® method.

Fraker and Speck introduced Iodogen® (Pierce Chemical Company, P.O. Box 117, Rockford, IL U.S.A. 61105), a non-aqueous insoluble reagent, to replace chloramine-T in order to eliminate the exposure of proteins to the soluble oxidizing agent during radioiodination (Fraker P.J. and Speck J.C., 1979). Chemically, Iodogen is 1,3,4,6, tetra-chloro-3α, 6α-diphenylglycouril (Fig. 8). Iodogen is insoluble in water, but it can be dissolved in chloroform or dichloromethane and coated on the inner surface of a reaction vial using a stream of nitrogen gas to evaporate the solvent (Markwell M.K. and Fox C.F., 1978). The appropriate amounts of protein and radioiodine are mixed and transferred into the iodogen coated

reaction vial. The reaction time and the amount of iodogen used varies from one protein to another, but generally the incubation time is in the range of two to five minutes (Perkins A.C. and Pimm M.V., 1991c) and the iodogen concentration used for one experiment may vary from 1 to 15 μg per 100 μg of protein (Regoeczi E., 1983). The reaction is terminated simply by removing the reaction mixture from the reaction vial.

Figure 8 The structure of Iodogen®. (Adopted from Markwell M.K., 1988).



Varying the amount of iodogen from 1 to 15 μg per 100 μg of protein or prolonging the incubation time from 0 to 15 min. was shown to affect the chemical integrity of the radioiodinated protein as measured by the ability of the intact radioiodinated protein to bind to Dowex 1-x 8 anion exchange resin (Regoeczi E., 1983). At low concentrations of

iodogen (1-5 μg/ 100 μg of protein) the incubation time did not show any effect, but as the concentration of the iodogen increased, the ability of the labeled protein to bind to the resin decreased. This effect is more pronounced as the incubation time increases. Therefore, high concentrations of iodogen and/or long reaction time do not improve radiolabeling efficiency, but may cause damage to the protein. On the other hand, increasing the protein concentration from 20 μg to 400 μg protein, keeping the other parameters unchanged, was reported to increase the radioiodine incorporation into the protein (efficiency) from 25% to 69% (Regoeczi E., 1983). Similarly, addition of carrier iodine (I^-) up to, an iodine-to-protein ratio of 5:1 was reported to increase the efficiency of radioiodine incorporation, but if the ratio exceeded this the efficiency decreased.

The advantages of this method are the simplicity, reproducibility and gentleness of the whole labeling procedure. Iodogen, as an oxidizing agent, gives higher iodine incorporation with less damage to proteins than the other commonly used oxidizing agents (Salacinski P., *et al.*, 1979).

2.5.2.2. The conjugation labeling method.

This method involves the attachment of radioiodine to some molecule other than the protein to be labeled, and conjugation of the radiolabeled molecule to the protein. Bolton-Hunter reagent (Bolton A.E. and Hunter W.M. 1972;

1973) and Wood reagent are popular examples of this method (Wood F. et al, 1975). Figure 9 shows the conjugation labeling reaction using Bolton-Hunter reagent and Figure 10 shows the radioiodination reactions using Wood reagent.

The Bolton-Hunter method is a two step reaction involving first the incorporation of radioiodine (*I) into N-succinimidyl-3(4-hydroxyphenyl)propionate in the presence of chloramine-T and then the conjugation of the iodinated propionate with the primary amino groups of the protein (Bolton A.E., 1977). This method provides the means of radioiodination of peptides which lack suitable tyrosine residues for direct iodination (Heymer B. *et al.*, 1975; Ciccimarra F. *et al.*, 1975). It also avoids the danger of protein damage as a result of exposure to oxidizing and reducing agents. The disadvantage of this method is that the technique is complex to perform, time consuming and causes more radiation exposure to the technician compared to the Iodogen method. The commercial availability of the radioiodinated propionate has minimized some of these drawbacks. The Wood reagent (Wood F. *et al.*, 1975) also plays the same role as Bolton-Hunter reagent except that in this case there are three steps involved in the protein labeling process. It has all of the advantages and the disadvantages of the Bolton-Hunter reagent.

Figure 9 Radioiodination of proteins with the conjugation of N-succinimidyl 3(4-hydroxyphenyl) propionate (Bolton-Hunter reagent) (Adopted from Bolton A.E., 1975).

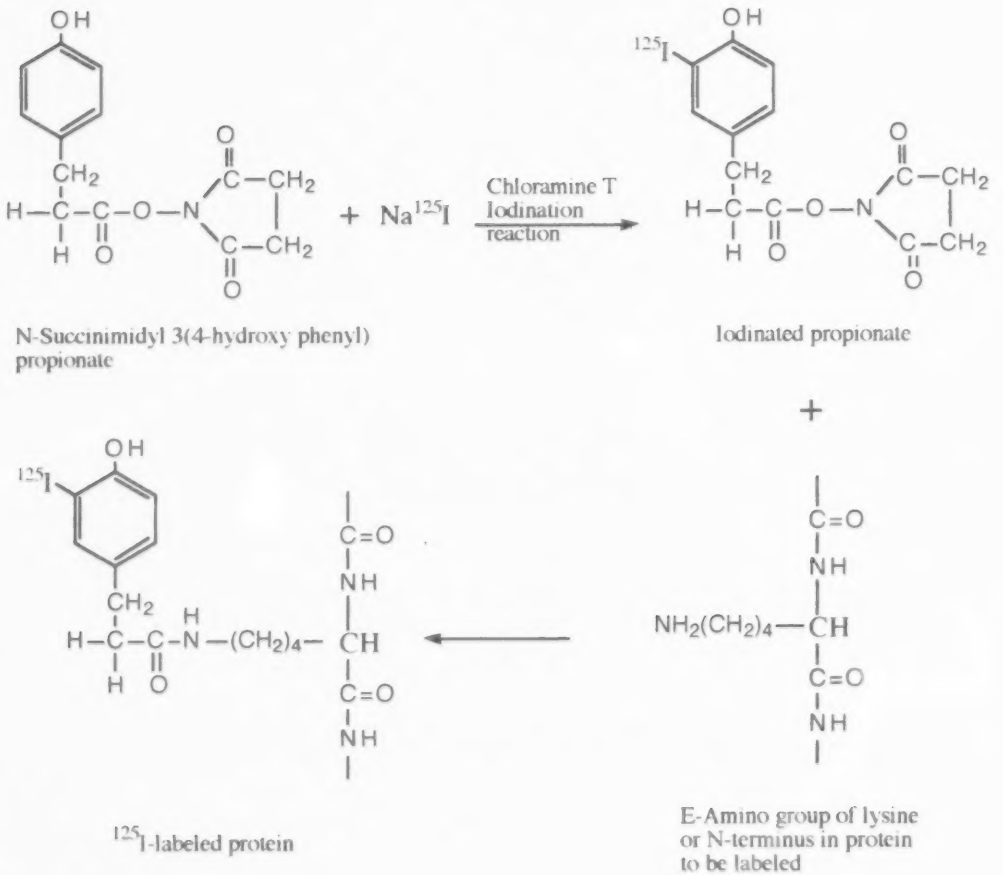
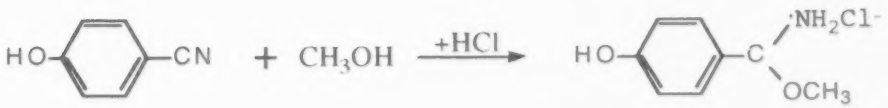


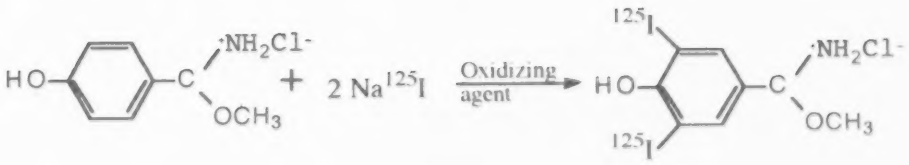
Figure 10 Radioiodination of proteins with the conjugation reaction of Wood reagent (Adopted from Wood F., *et al*, 1975).

1.

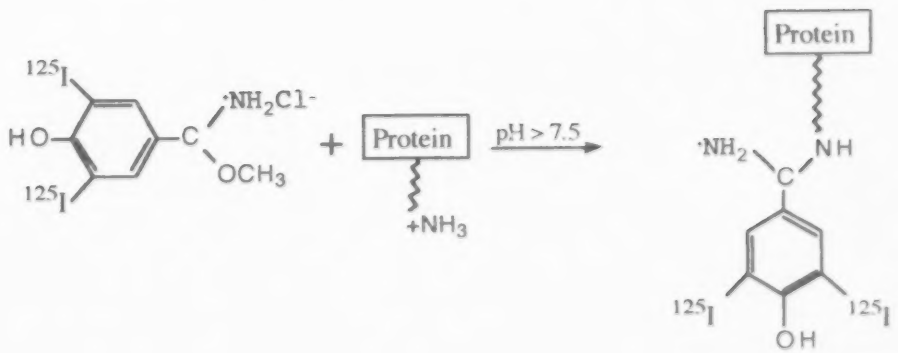


(MPHBIM)

2.



3.



2.6. The QUALITY CONTROL OF RADIOLABELED MONOCLONAL ANTIBODIES.

The quality control of radiolabeled monoclonal antibodies intended for clinical application involves: 1) the radionuclidic and radiochemical quality control, 2) the immunological quality control and 3) the sterility, apyrogenicity and toxicity tests .

2.6.1. The Radionuclidic and the Radiochemical Quality Control.

The radionuclide to be used for the labeling of monoclonal antibody must be checked for its identity and purity. The identification of the radionuclide can be performed by the following methods:

1) the determination of the half-life of the radioisotope using the radiation decay curve method (Chase G.D. and Rabinowitz J.L., 1967).

2) the determination of the type of radiation emitted and its energy.

3) the comparison of the results in 1 and 2 with reference sources and chart of spectra.

The radiochemical purity can be defined as the proportion or percentage of the total radioactivity that is present as the stated chemical form of the radiopharmaceutical. The radiochemical quality may be verified by one or a combination of the following methods:

1) chromatographic method; including instant thin layer chromatography (ITLC) (Billinghurst M.W., 1973;

Sundrehagen E., 1982), high performance liquid chromatography (HPLC) and soft gel size exclusion chromatography techniques (Hnatowich D.J., 1986), ion exchange chromatography e.g. diethylene aminoethyl (DEAE) cellulose chromatography, (Parham P., *et al.*, 1982; Bruck C., *et al.*, 1986) and affinity chromatography e.g. Protein A and Protein G chromatography systems (Sundrehagen E., 1983; McGuire J., 1989).

2) precipitation method using trichloroacetic acid (TCA) (Shen V. *et al*, 1974).

3) electrophoresis method (Chase G.D. and Rabinowitz J.L., 1967).

4) animal *in vivo* tests whereby the radiochemical purity may be determined by checking its localization in the various organs of an animal model using whole-body scintigraphy and/or biodistribution methods. In ^{99m}Tc-labeled radiopharmaceuticals, colloidal radiochemical impurities may be detected by the localization of radioactivity in the liver and the other reticulo-endothelial systems. $^{99m}TcO_4^-$ is another radiochemical impurity which may be detected by its localization in organs such as thyroid, stomach and choroid plexus in the brain.

2.6.2. The Immunoreactivity Quality Control of Radiolabeled Monoclonal Antibodies.

The immunointegrity quality control is a measure of the degree of immunoreactivity of the radiolabeled monoclonal antibody with the antigen of interest. The methods used to determine the degrees of immunoreactivity include:

1) Enzyme-Linked Immunosorbent Assay (ELISA) (Kemeny D.M. and Chantler S., 1988).

2) Whole-cell ELISA, which is a standard ELISA method that uses cells grown in or attached (fixed) to the well of microtiter plates as source of antigen (Turner C.J., 1986).

3) Live cell uptake, which uses live cells in suspension to determine the percent uptake of radioactive monoclonal antibody by the cells (Turner C.J., 1986).

4) Radioactive binding assay where the amount of radioactive antibody bound to the antigen is determined as a fraction of the total radioactivity used. The result gives the degree of the immunoreactivity of the radiolabeled monoclonal antibody with the antigen (Turner C.J., 1986).

5) Histological assay involves cellular antigens in tumor tissue sections that can be used to determine the immunoreactivity of radiolabeled monoclonal antibodies using the immunoperoxidase method of staining (Imam A., *et al*, 1986).

6) animal *in-vivo* localization test may be used to

evaluate the quality of a radiolabeled monoclonal antibody preparation. This is performed by using whole-body radioimmunoimaging technique and/or by determining the biodistribution of the radiolabeled antibody in the critical organs including the tumor.

2.6.3. Sterility, apyrogenicity and toxicity tests.

Radiolabeled monoclonal antibody preparations intended for human use must be free from any bacterial contamination. The test for sterility is done on samples from each batch of preparations. Detailed information on the method employed for sterility tests are described in the United States Pharmacopoeia (USP XXII, 1990a).

Radiopharmaceuticals are also required to be free from the presence of pyrogens. Pyrogens are lipopolysaccharides produced by gram-negative bacteria. There are two methods of testing the presence of pyrogens in radiolabeled monoclonal antibody preparation. They are:

1) Rabbit test, which is based on the fever response of the rabbit, within 3 hours after injection of the preparation. Detailed information on the rabbit test is available in the USP XXII, (USP XXII, 1990b).

2) the Limulus Amebocyte Lysate (LAL) test for pyrogens is a much simpler and quicker test and is preferable for radiopharmaceuticals with short half lives (Yin E.T., *et al*, 1972; Tubis M., 1976).

To insure that the product is safe for human use it is also important to perform toxicity tests on radiolabeled

monoclonal antibody preparation. The method uses mice injected with an aliquot of the preparation to determine its toxicity. The Food and Drug Administration (FDA) require as a minimum that the toxicity test, such as the determination of LD_{50} and histological tests on various organs, be done in two species of animals including a non-rodent such as dog (FDA of U.S.A., 1971).

3. MATERIALS AND METHODS.

3.1. SOURCE OF MATERIALS USED.

All of the radioisotopes used were supplied by the Edmonton Radiopharmacy Centre, Edmonton Alberta. Chemicals used were reagent grade unless otherwise specified. Microtiter plates for the ELISA work were purchased from Gibco® Inc., Burlington Ontario. The following materials antibodies were gifts from Biomira Inc. Edmonton, Alberta: MAbs 174H.64, 170H.82 and B80.3 and the human tumor tissues. The bovine tumor tissues were acquired fresh from a slaughter house in Edmonton. Immediately after the collection the tissues were trimmed, packed, labeled and snap-frozen in isopentane at -80^{o}C and stored frozen at -80^{o}C ready for use. All of the tumor cell lines used were obtained from Biomira Inc. Edmonton, Alberta. The rabbits and the mice used for the *in vivo* studies were purchased from the Health Science Small Animal Program, University of Alberta.

3.2. METHODS

3.2.1. Screening of Tumor Tissues and Cell Lines for Squamous Cell Typing and for Their Immunoreactivity with MAb174H.64.

3.2.1.1. Immunohistological screening.

Immunoperoxidase staining of frozen tumor tissue sections and cell smears were performed by Dr. G.S. Brzezinska of Biomira Inc. Edmonton. The method of screening is according to the Avidin-Biotin Complex (ABC) method provided by Biomira Inc. Edmonton and is described here. Fresh human or bovine

tumor tissues were embedded in OCT® medium and snap-frozen in isopentane at -80°C. The frozen sections were cut into ≈ 8μm thick slices and placed directly on glass slides coated with poly-L-lysine and stored at -80°C for future use. Cells grown in culture, after being assayed for viability, concentration and total cell count, were washed and resuspended in phosphate buffered saline (PBS) at a concentration of 2 x 10^6 cells per ml. The cells were then placed on poly-L-lysine coated glass slides in aliquots of 100 μl. Subsequently they were dried and stored at -70°C ready to use for up to 8 weeks.

The frozen sections and the cell preparations mounted on glass slides were washed in PBS for 5 minutes before each incubation step. MAb174H.64 was added to the frozen sections and cell preparations and allowed to react for 15 minutes. The second antibody (Biotinylated horse antimouse IgG, Sigma chemical Co. St. Louis MO 63178 U.S.A.) was then added and incubated for a further 30 minutes during which time it reacted with antigen bound primary MAb. In the next step, avidin-peroxidase conjugate was added and incubated on the slide for 20 minutes to allow reactions with the antigen-MAb174H.64-biotinylated antimouse IgG complex. Subsequently the substrate, 3,3' diaminobenzidine (Sigma Chemical Co. St. Louis MO 63178 U.S.A.), was added to form an insoluble color dye on the sites of the original reaction between the antigen and MAb174H.64. The slides were then counter-stained with hematoxylin, dehydrated and mounted. The intensity of stained tumor cells and the percent of total tumor cells stained were

evaluated using light microscopy and graded on the scale of 0 to 4 and 0 to 100% respectively. The product of these two measurements on each tissue type and cell line were given as an Immunoreactivity Unit (IRU). Control studies were done repeating the same procedure as stated above but using MOPC-21 (Sigma Chem. Co. St. Louis, M0 63178) in place of MAb174H.64. MOPC-21 is a mouse myeloma protein (IgG_1 κ), which is non-antigen reactive. During hybridomas between myeloma cells and antibody secreting splenocytes, the myeloma cell antibody chains are combined to form the myeloma cell Ig called MOPC (McCullough K.T. and Spier R.E., 1990f). This unique property has made MOPC-21 very useful as a control agent.

3.2.1.2. Radio-immunoreactivity screening.

The radio-immunoreactivity screening process was performed using ^{99m}Tc labeled MAb174H.64 and various tumor cell lines in culture. The radiolabeling of MAb174H.64 with ^{99m}Tc was performed according to the following procedure. Aliquots of 100 μl of pretinned MAb174H.64 solution (3.0 mg/ml), obtained from Biomira Inc. Edmonton, were directly mixed with 200 μl of Na $^{99m}TcO_4$ (1.5 GBq/ml). The mixture was incubated for about 15 minutes with occasional gentle swirling. The radiolabeling efficiency was determined by either size exclusion HPLC or ITLC (85% methanol as solvent)as described in section 3.2.5. hereinafter.

Tumor cell lines from SiHa (a human uterine cervix SCC), FaDu (a human pharynx SCC) and KLN-205 (a mouse SCC) were selected for this study because they represented SCC cell lines from diversified origins. The cell lines were supplied by Biomira Inc. Edmonton, Alberta. The cells in culture were centrifuged at 1500 RPM for 3 minutes and the cell pellets were washed with 500 μL of PBS. The final suspension of each cell line in PBS were prepared in the following batches of concentration: 0.25×10^6, 0.5×10^6, 1.0×10^6, 2.0×10^6 and 3.0×10^6 cells each in 150 μL of PBS. Each batch of the cell preparation was mixed with 5 ng of ^{99m}Tc174H.64 ($\approx 1.0 \times 10^4$ cpm) in a total volume of 200 μL and incubated for one hour at room temperature (22°C) with occasional gentle mixing. Then 1 mL of sterile PBS in 1% BSA was added to the above mixture and centrifuged at 1500 RPM for 5 minutes. The cell pellets were washed with 500 μL of the PBS in 1% BSA and again centrifuged at 1500 RPM for 5 minutes. The cell pellets and the washes were assayed for the amount of radioactivity in each. The radioactivity in the cell pellets was indicative of the total cell bound-radioactivity.

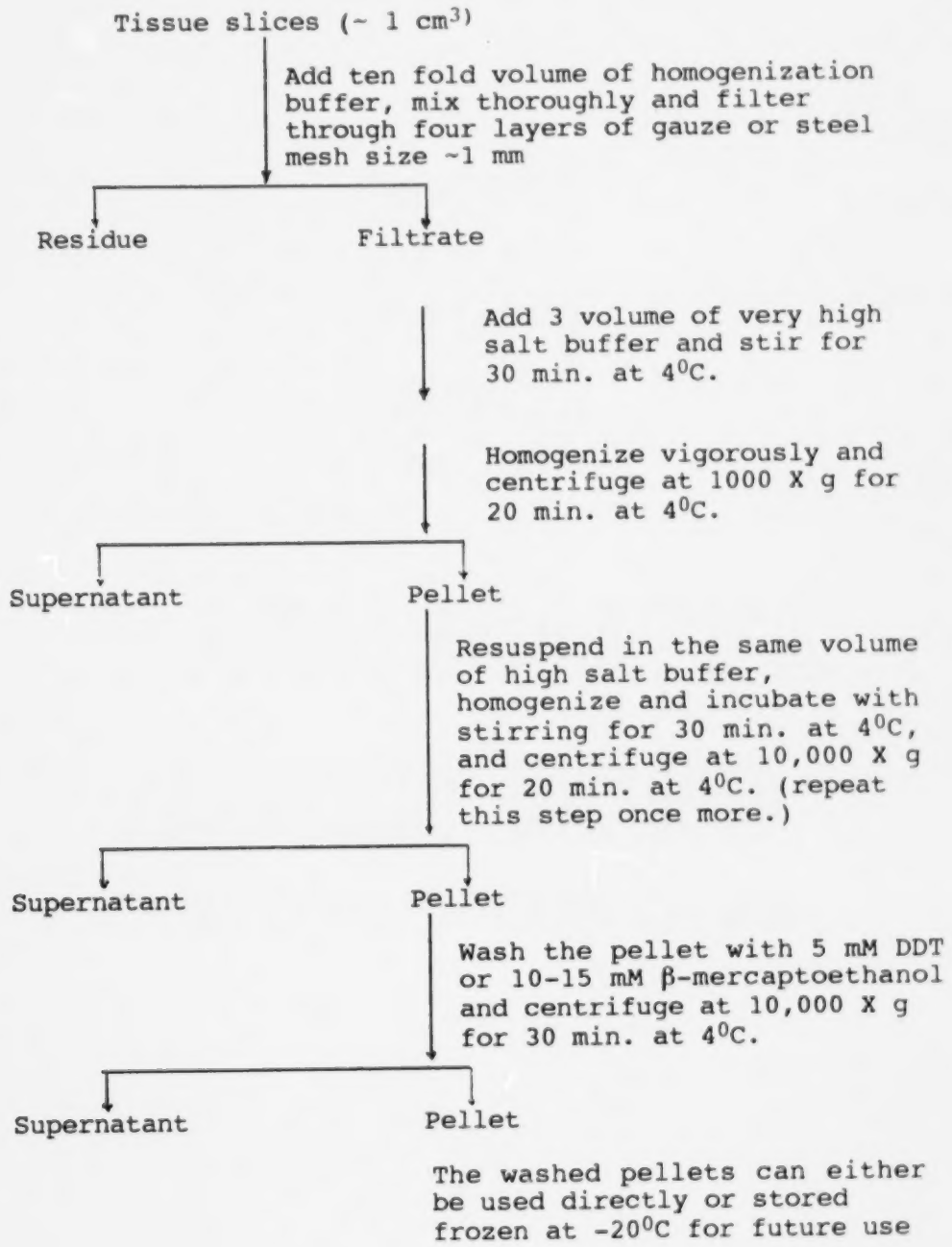
3.2.2. Extraction, Purification and Characterization of Cytokeratin Proteins.

The tumor tissues and the tumor cell lines which showed immunoreactivity with MAb174H.64 by immunoperoxidase staining method were selected. Protein extracts were prepared, according to a reported procedure (Achtstaetter T., *et al.*, 1986), from the bovine SCC tissue and SCC cell lines (FaDu,

and KLN-205) which were provided by Biomira Inc. Edmonton. These cell lines were selected because they all showed strong staining during the immunohistological screening. The extraction of the cytokeratin proteins from the various tissue is presented in Figure 11 and also described in the following paragraphs.

Tissue samples were homogenized in a Potter-Elvehjem glass tissue grinder (pestle clearance 0.15 mm, Fisher Scientific, Edmonton, Alberta) with a 10 fold volume of homogenization buffer (96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2PO_4 $2H_2O$, 1.5 mM KCl, 10 mM EDTA, 0.1 mM DTT pH 6.8) at 4°C for 30 min. and then filtered through four layers of gauze or steel sieve (mesh size ~1 mm). Three volumes of a very high salt buffer (2.0 M KCl, 200 mM NaCl, 10 mM tris HCl, 0.1 mM DTT pH 7.4) were added to the filtrate. This was stirred for 30 minutes at 4°C and homogenized vigorously using the homogenizer and then centrifuged for 20 min. at 10,000 x g at 4°C. The pellets were resuspended in the same volume of high salt buffer (10 mM Tris HCl, 140 mM NaCl, 5 mM EDTA, 0.5% (w/v) triton X-100, 5 mM dithiothreitol (DTT) pH 7.6), homogenized, incubated for 30 min. at 4°C with constant stirring and then centrifuged for 20 min. at 10,000 x g at 4°C. After the last step was repeated, the pellets were resuspended in phosphate buffered saline (PBS) containing 5 mM DTT and centrifuged at 10,000 x g at 4°C for 20 minutes. The final pellet prepared in this way could be used directly or be stored at -20°C for future use.

Figure 11 Flow chart for cytokeratin extraction from SCC tumor tissue. (Adopted from Achtstaetter T., *et al.*, 1986).


Tissue slices (~ 1 cm3)
Add ten fold volume of homogenization buffer, mix thoroughly and filter through four layers of gauze or steel mesh size ~1 mm
Residue
Filtrate
Add 3 volume of very high salt buffer and stir for 30 min. at 4°C.
Homogenize vigorously and centrifuge at 1000 X g for 20 min. at 4°C.
Supernatant
Pellet
Resuspend in the same volume of high salt buffer, homogenize and incubate with stirring for 30 min. at 4°C, and centrifuge at 10,000 X g for 20 min. at 4°C. (repeat this step once more.)
Supernatant
Pellet
Wash the pellet with 5 mM DDT or 10-15 mM β-mercaptoethanol and centrifuge at 10,000 X g for 30 min. at 4°C.
Supernatant
Pellet
The washed pellets can either be used directly or stored frozen at -20°C for future use

A milder procedure was used to extract cytokeratin protein from the SCC cell lines. Cells separated from culture media were washed with PBS and centrifuged at 10,000 x g. The pellets were then mixed with detergent buffer consisting of 10 mM tris HCl, 140 mM NaCl, 5 mM EDTA, 1% triton X-100, 5 mM DTT, pH 7.6; incubated for 5 minutes and centrifuged for 20 minutes at 10,000 x g at 4°C. The pellets were then incubated in the high salt buffer solution for 30 minutes with gentle swirling. The final pellets, after being washed with PBS and centrifuged under the same condition as above, were stored at -20°C until utilized.

To determine the molecular weights of the cytokeratin proteins present in the extract the SDS-PAGE electrophoresis method was used. The detailed procedure is available in the Phast-gel® electrophoresis manual (Pharmacia®s-75182 Uppsala 1, Sweden). The cytokeratin pellet samples were dissolved in 9 M urea and mixed with 2.5% SDS, 5% ß-mercaptoethanol and 1% bromophenol blue. The mixtures were boiled in a water bath at ≈ 100° C for 5 minutes, centrifuged at 1000 RPM for 2 minutes and then 1 μL of the supernatants were applied on SDS-PAGE 12.5% homogeneous gels (Pharmacia® s-75182 Uppsala 1, Sweden). A low molecular weight standard (range of 14 kd to 94 kd) was also prepared in the same way as that for the samples. The electrophoresis was run using a Phast-Gel electrophoresis system (Pharmacia®s-75182 Uppsala 1, Sweden), according to the procedure outlined in the manufacturer's manual. Coomassie blue was used for staining. The molecular

sizes for the component proteins in the samples were determined from a molecular weight standard curve in which the Rf values were plotted as a function of the molecular weight of the standards.

3.2.3. The Specificity of the Immunoreactivity of the Cytokeratin Protein, with MAb174H.64.

3.2.3.1. The ELISA method of analysis.

Cytoskeletal protein extracts from the following tissues and cell lines were used as sources of antigen for the ELISA analysis:

a) Tissues form bovine SCC and adenocarcinoma tumors.

b) Cells in culture from FaDu and KLN-205 cell lines.

The following monoclonal antibodies were used in the ELISA assay: MAb174H.64, MAb B80.3, 170H.82 (Biomira Inc., Edmonton, Alberta) and MOPC-21 (Sigma Chem. Co. St. Louis, M0 63178). The tissue and cell extracted cytokeratin proteins were coated for 16 hrs on microtiter plates (10 μg/100 μL/well). Then after washing with PBS and blocking for 1 hr. with 200 μL/well of 5 % fetal calf serum (FCS), the monoclonal antibodies 174H.64, 170H.82 and B80.3 and the control MOPC-21 (2 μg/100 μL/well) were added into each well separately to be incubated for 3 hrs. Then after thorough washing with PBS, 100μL/well of a goat anti-mouse IgG peroxidase conjugate (Sigma Chem. Co. St. Louis M0 63178) was added and incubated for 1.5 hrs. Finally, after another thorough washing of the microtiter plates, 100 μL/well of a

freshly prepared ABTS (stock solution) and hydrogen peroxide (stock solution) mixture (1:1 v/v) were applied to the wells and incubated for 20 minutes. Color development in each well was determined by measuring optical density (OD) utilizing a micro plate reader (MR600, Dynath Instruments Inc., Torrence, California). The specificity of the MAb174H.64 was determined by comparing the OD of samples in the well containing the SCC extracted cytokeratin with that of the control studies.

3.2.3.2. The competitive binding assay.

One of the freeze stored SCC cytokeratin protein pellets, from a batch which were tested earlier to have strong immunoreactivity with MAb174H.64, was allowed to thaw and was then dissolved in 6 M guanidine HCl. A microtiter plate was coated with the cytokeratin protein solution in 6 M guanidine HCl (10 μg/well) and incubated for 16 hr, followed by washing with PBS. Then 200μL of 3% BSA in PBS was added and incubated in each of the wells of the microtiter plate for 1 hr and washed with PBS This blocks the non-specific binding sites. Mixtures of 100 ng (200,000 CPM) ^{99m}Tc174H.64 and different concentrations of non-radioactive MAb174H.64, ranging from 0.63 μg to 50 μg, were incubated in the wells in triplicate for 2 hrs. Finally, the wells were thoroughly washed with PBS and the radioactivity in each well was determined using a Beckman-8000 gamma counter (Beckman Industries Inc. Fullerton CA 92634, U.S.A.).

3.2.4. Preparation, Purification and Characterization of F(ab')$_2$ Fragments of MAb174H.64 (IgG).

The MAb174H.64 (IgG) was provided by Biomira Inc. Edmonton at a concentration of 3.5 mg/mL in phosphate buffer. The materials and method used for the preparation of the F(ab')$_2$ fragment was as described in the Pierce ®(Pierce Chemical Co. P.O.Box 117, Rockford, IL., U.S.A. 61105) technical manual for their Immunopure® F(ab')$_2$ preparation kit. In this procedure, the MAb174H.64 in solution was dialyzed against a 20 mM phosphate/10 mM EDTA buffer at pH 7.0 overnight. The solution was concentrated to an IgG concentration of approximately 20 mg/mL. Then 0.5 mL of the dialyzed solution was mixed with 0.5 mL of freshly prepared digestion buffer (42 mg cysteine-HCl in 12 mL of phosphate buffer). The mixture was added into a test tube containing immobilized pepsin (Pierce Chemical Co. P.O.Box 117, Rockford, IL., U.S.A. 61105) and incubated for 8 hours at 37^{o}C on a shaker at high speed. The solubilized F(ab')$_2$ and Fc fragments and the undigested IgG (crude digest) were recovered from the immobilized pepsin gel using a separator tube. Finally the F(ab')$_2$ fragment was purified first by allowing the crude digest to pass through a Protein A® column (Pierce Chemical Co. P.O.Box 117, Rockford, IL., U.S.A. 61105) using binding buffer (pH 8.8) as elution solution, followed by dialyzing the eluate using a dialysis membrane with a molecular weight cut-off point of 50 kilo-dalton, against PBS buffer solution for overnight at 4^{o}C.

The final purified $F(ab')_2$ fragment was analyzed to characterize its molecular weight using SDS-PAGE non-reducing electrophoresis. The method is as described in section 3.2.2. with the following modification. At the sample preparation stage, no addition of ß-mercaptoethanol or heating the sample were done.

3.2.5. Radioiodine or ^{99m}Tc Labeling and Quality Control of Monoclonal Antibodies (MAbs).

3.2.5.1. Radionuclidic identity.

The radionuclidic purity of the radioiodine solution, $Na^{125}I$, $Na^{131}I$ and the ^{99m}Tc sodium pertechnetate (Edmonton Radiopharmaceutical Center, Edmonton, Alberta) was determined by analyzing the major photopeaks of the isotopes using a calibrated multichannel analyzer (Cambera model 7411 Cambera Industries Inc. 45 Gracey Ave. Meriden Con. 06450 U.S.A.) and comparing it with standard literature values.

3.2.5.2. Radiochemical purity

The radiochemical purity of each of the radiolabeled proteins was determined by utilizing the instant thin layer chromatography (ITLC) and/or size exclusion HPLC analysis method. In the ITLC method strips (20 cm) of ITLC SG Gelman® (Gelman, Ann. Arbor Michigan, U.S.A.) were spotted with approximately 20 kBq of each solution, air dried and allowed to develop for about 15 cm in 85% methanol in water. The strips were cut into 1 cm segments and placed separately in test tubes and counted in a Beckman 8000 gamma counter. The

HPLC method is as described in section 3.2.5.6.3.

3.2.5.3. Radioiodination of monoclonal antibodies.

Monoclonal antibodies were iodinated with ^{131}I or ^{125}I by the Iodogen method described originally by Fraker P.J. and Speck, J.C., 1979, with slight modifications. In the procedure, 2 μg of Iodogen (1,3,4,6, tetrachloro-3-α-6-α-diphenylglycouril) (Pierce Chemical Co. P.O.Box 117, Rockford, IL., U.S.A. 61105) was dissolved in 20 μL of analytical grade chloroform and coated onto the bottom of a 12 x 75 mm test tube, using a gentle stream of nitrogen to evaporate the chloroform. The required amount of the MAb to be iodinated was mixed with the appropriate quantity (40 -300 MBq) of Na ^{125}I or $Na^{131}I$ solutions in 0.1 M NaOH, which had been buffered to pH 7.4 with PBS. The radioiodine-MAb mixture was transferred into the iodogen-coated tube and incubated for 2 minutes with occasional gentle swirling. The reaction mixture was then transferred into another test tube which contained 20 μL of 1.0 M NaI solution and incubated for 20 minutes. Finally, the radioiodinated antibody was separated from the free (unreacted) radioiodine present in the reaction mixture by using the size exclusion chromatographic technique as described in section 3.2.5.4.

3.2.5.4. Purification of the radioiodinated monoclonal antibody.

The purification process was performed using size exclusion gel chromatography. A Bio-gel® P-100 (Bio-Rad Lab. Mississauga Ontario) with a size exclusion limit of 100,000 daltons was swollen for at least 24 hours in PBS and packed into a 1.5 x 25 cm glass column by gravity. The non-specific binding sites on the gel were saturated by passing 1.0 mL of 1 % BSA in PBS through the column and washing with 10 mL of PBS. The reaction mixture was then applied onto the column gel bed and allowed to drain in. A small volume of PBS was used to wash the mixture into the gel bed. The elution profile was monitored by uv absorbency at 280 nm and radioactivity detectors, for the detection of the radioiodinated protein and the free radioiodine. A uv monitor (LKB 2238 UVICORD SII, Pharmacia, s-75182 Uppsala 1, Sweden.) was set at 280 nm wavelength, with the base line adjusted to zero using PBS as blank. A 3"x 3" NaI (Tl) crystal detector coupled to a single channel analyzer was used to detect the radioactivity in the eluate. The column was eluted with PBS (pH 7.4) and aliquots of 1 mL were collected in glass test tubes. The elution profile from the uv monitor and the NaI detector were recorded on a chart recorder to show the fractions containing protein and radioactivity. The amount of radioactivity in each of the fractions collected was determined using an isotope dose calibrator. The protein fractions corresponding to the high radioactivity peak were

pooled and mixed thoroughly. This method of purification is relatively simple but the time required to complete the separation is about 30 min. to one hour. The other disadvantage of this method is the considerable sample dilution. This can be a serious problem if high specific activity in a small volume is required as in the case of biodistribution studies. This method was employed as a quality control rather than production method.

The method used here for the separation of radioiodinated MAbs from the rest of the preparation mixture was the centrifuged column technique. The column used was Bio-gel® P-100 packed in 10 mL volume mini-column (Bio-Rad Lab. Mississauga Ontario). The non-specific binding sites on the gel were saturated by passing 1.0 mL of 1 % BSA in PBS through the column and washing with 2 mL of PBS followed by centrifugation at 100 x g for 2 minutes. The reaction mixture was then applied onto the column gel bed and allowed to drain in and centrifuged at 100 x g for 2 minutes.

3.2.5.5. Labeling of monoclonal antibodies with ^{99m}Tc pertechnetate.

The labeling of monoclonal antibodies with ^{99m}Tc was performed by the direct labeling method. In this process the monoclonal antibody was first incubated with stannous ions for about 24 hours (pretinning) to reduce the disulfide groups in the antibody, then ^{99m}Tc was introduced into the mixture. The excess stannous ions present in the mixture were also utilized for the reduction of the Tc^{+7}. The detailed

pretinning procedure is a proprietary process of Biomira Inc. Generally, the procedure of mixing the pretinned antibody with ^{99m}Tc proceeds in the following ways. One hundred microliters of the pretinned MAb174H.64 (3.0 mg/mL) was allowed to mix with 200 μL of $^{99m}TcO_4^-$ (1.5 GBq/mL) for 15 minutes with occasional swirling. The radiolabeling efficiency was determined by applying a tracer amount of the preparation on to a strip of ITLC-SG paper and developing it with a 85% v/v methanol in water solvent system. The ITLC strip was cut into 1 cm length segments and the radioactivity in each was determined using a Beckman 8000 gamma counter. Size exclusion HPLC was also used to determine the labeling efficiency of the preparation as described in the following section.

3.2.5.6. Quality control of radiolabeled monoclonal antibodies.

In the preparation of radiolabeled whole-monoclonal antibodies there are undesirable radiolabeled products which are termed as radiochemical impurities. These include free radionuclide and radiolabeled antibody fragments. When the preparation is for radiolabeled $F(ab')_2$ fragment the radiochemical impurities may be free radionuclide, radiolabeled whole-antibody and radiolabeled Fab fragments. However in the preparation of ^{99m}Tc labeled monoclonal antibody through the $SnCl_2$ reduction method, colloidal radionuclide formation is also a possible impurity. Therefore

the quality control of radiolabeled monoclonal antibodies requires all or a combination of the following methods.

3.2.5.6.1. The trichloroacetic acid (TCA) precipitation method.

The percent protein-bound radioactivity was determined by the trichloroacetic acid precipitation method. The method is as following: aliquots of the purified radiolabeled MAb (2 to 5 μL) were diluted with 0.5 mL of 1% BSA in PBS in an Eppendorf® tube (Pierce, P.O.Box 117, Rockford, Il. 61105, U.S.A.), to each of which 0.5 mL of 25% TCA was added. The resulting denatured protein was mixed well on a vortex mixer and allowed to stand for ten minutes. Then the mixture was centrifuged for 10 minutes at 1000 x g. The precipitate was washed twice, with 0.5 mL PBS followed by centrifugation at 1000 x g. The washed precipitate as well as the pooled washes were counted for radioactivity. The percent protein bound radioactivity was calculated as a ratio of the CPM in the pellets to the total CPM in all fractions. This method only indicates what fraction of the total radioactivity is present as protein bound or free unbound radionuclide.

3.2.5.6.2. The instant thin layer chromatography (ITLC) method.

The radiolabeled MAb (20 μL aliquot) was spotted on to a 20 cm ITLC SG strip (Gelman Science Inc., Ann Arbor MI, 48106, U.S.A.), allowed to air dry and then developed in an appropriate solvent system. The different solvent systems used were: 1) 85% v/v methanol in water; 2) 20% v/v acetone

in water and 3) 10% w/v ammonium sulfate in water, 20% v/v methanol and water, in the ratio of 2:6:4. After the developed strips were dried, they were cut into 1 cm segments and the radioactivity in each was determined using a Beckman 8000 Gamma counter. This method of analysis is expected to show the radionuclide bound to the protein and the free unbound radionuclide.

3.2.5.6.3. HPLC method.

HPLC analysis was made on samples of ^{99m}Tc labeled monoclonal antibody preparations. The HPLC used was a TSK®-GEL size exclusion column type G3000SW (Phenomenex, 6100 Palos Verdes Drive So., Rancho Palos Verdes, CA, 90274-5935, U.S.A.). A pre-column was attached to the main column in order to prevent the passage of any minute particulate matter into the main column. Each sample was filtered through 45 nm Millipore filters and an aliquot of 25 μL was applied to the column. The elution buffer was prepared from a mixture of 1.0 M NaH_2PO_4, 1.0 M Na_2SO_4 and sodium azide with pH adjusted to 7.2. The flow rate was adjusted to 0.1 mL per minute. The HPLC was attached to a uv monitor and a NaI (Tl) detector to determine the presence of protein and radioactivity, respectively. The response of the detectors was recorded on charts displaying optical density or radioactivity versus retention time. Elution fractions were collected every 30 seconds.

3.2.5.6.4. ELISA quality control of the immunoreactivity of the radiolabeled MAb174H.64.

Cytokeratin protein obtained from bovine SCC eyelid tumor tissue in 4 M guanidine HCl solution (10 μg/100 μL/well) was coated on microtiter plates (Gibco® Canada Inc. Bellington, Ontario) and incubated at about 20°C for 16 hrs. After washing twice with PBS and blocking for 1 hr with 200 μL/well of 5% fetal calf serum (FCS), 2 μg/100 μL/well of the radiolabeled MAb174H.64 or unlabeled MAb174H.64 (control) were incubated for 3 hrs in the coated wells. Then following three thorough washings with PBS, 100 μL/well of goat anti-mouse IgG peroxidase conjugate (Sigma Chemical Co., St. Louis Mo. 63178 U.S.A.) diluted 1:1000 with PBS was added and incubated for 1.5 hrs. Finally after another three thorough washings of the microtiter plates with PBS, 100 μL/well of freshly prepared ABTS and hydrogen peroxide mixture (1:1 v/v) were incubated in each well. Color development in each well was determined by measuring the optical density (OD) using a microtiter plate reader. The immunoreactivity of the radioiodinated MAb174H.64 was determined by comparing the OD of the sample with that of the control.

3.2.6. The Radiochemical and Immunological Stability of Radiolabeled MAb174H.64.

3.2.6.1. Incubation of ^{99m}Tc174H.64 in various body fluids.

The effect of incubation of ^{99m}Tc174H.64 in blood, plasma and urine can be evaluated by considering the following parameters. 1) The radiochemical purity of the radiolabeled antibody which incudes: a) the integrity of the antibody, intact form or fragmented and b) the stability of the radiolabel and 2) the preservation of the immunoreactivity of the antibody.

Protein A chromatography is a useful method to analyze and determine the percent radiolabeled whole-IgG in a solution. IgG from mammalian species have high affinity with Protein A. Protein A is a cell membrane protein obtained from most strains of *staphylococcus aureus* (Sundrehagen E., 1983). The interaction of Protein A with IgG is mediated through the Fc-portion of the IgG molecule. By immobilizing Protein A in a gel, separation of intact IgG from its fragments may be obtained. Hence the chemical purity of ^{99m}Tc174H.64 (IgG) can be defined as the fraction of ^{99m}Tc that is bound to the IgG molecules and which reacts with protein A.

The protein A column used for this analysis was prepared from about 2 mL of Protein A agarose gel slurry (Pierce Chemical Co., P.O. Box 117, Rockford, IL U.S.A. 61105). The gel was packed in to a 5 mL Pierce disposable polystyrene column. The binding capacity of the protein A is 6-8 mg of

mouse IgG per mL of the gel slurry. For optimal recovery it is recommended that sample size should be less than 80% of the maximum binding capacity.

Aliquots of ^{99m}Tc174H.64, 700 μCi (25.9 MBq)/22 μg /40 μL, were mixed with 3 mL of each of the body fluids. These concentrations were based on conditions projected for human use. The mixtures were incubated for 1 hr at 37°C. The fluids were collected fresh from a healthy volunteer. Samples containing about 1.5 μg/46.6 μCi (1.72 MBq)/200 μL of ^{99m}Tc174H.64 from each of the above preparations were mixed with 800 μL of binding buffer (0.1 M glycine and 3 M NaCl pH 8.9) and then separately applied to Protein A columns. The column was pretreated with 1% BSA to block the non-specific binding sites and washed with the binding buffer, before the samples were applied to the column. ^{99m}Tc174H.64, 45 μCi (1.66 MBq)/1.5μg/10 μL was mixed with 200 μL of the binding buffer and applied to the Protein A column as a control. The columns were each eluted with the binding buffer to obtain the unbound fraction (UBF) and then eluted with elution buffer (100 mM citric acid pH 3) to remove the bound fraction (BF). The radioactivity in each of the UBF, the BF and that retained in the column, were determined using a Beckman 8000 gamma counter. The amount of free pertechnetate in each of the UBF and BF was determined using ITLC with 85% v/v methanol in water as solvent.

ELISA was performed on the ^{99m}Tc174H.64 incubated in the various body fluids. The procedure is as described earlier in

section 3.2.5.6.4. The ^{99m}Tc174H.64 (2 μg/100 μL/well), which was incubated in PBS, was used as a positive control antibody and body fluids without antibody were used as negative control.

3.2.6.2. Incubation of ^{131}I-174H.64 in the various body fluids.

The stability of ^{131}I-174H.64 incubated in the different body fluids, was performed in the same way as that described above for the ^{99m}Tc174H.64. Aliquots of 200 μl of ^{131}I-174H.64, 300 μCi (11.1 MBq)/3 mL of each of the body fluids, were mixed with 800 μl of the binding buffer. Another aliquot of 10 μL of ^{131}I-174H.64, 30 μCi (1.11 MBq)/10 μL, which was incubated in PBS, was mixed with 200 μL of the binding buffer and used as a control. Then the mixtures were applied separately to different Protein A columns, and eluted with the binding buffer followed by elution buffer as UBF and BF respectively. The radioactivity in each of the UBF and BF was determined and the percent free radioiodide in each were analyzed using the ITLC method with 85% v/v methanol in water as solvent.

ELISA was performed on the ^{131}I-174H.64 incubated in the body fluids in the same way as that described for ^{99m}Tc174H.64 in the preceding paragraph.

3.2.6.3. Effects of temperature on the immunological stability of MAb-174H.64.

Aliquots of 300 µl containing about 1 µg /µL of MAb174H.64 were incubated for 30 minutes in water bath at temperatures of 20°C, 37°C, 60°C, 80°C and ≈100°C, with occasional mixing. The immunological stability of the monoclonal antibody was determined using the ELISA method as described earlier in section 3.2.5.6.4. A non-heat-treated MAb174H.64 was used as a positive control.

3.2.6.4. Effects of Iodogen® on the immunological stability of MAb-174H.64.

Various concentrations of iodogen (Pierce Co., P.O.Box 117, Rockford, Illinois 61105) in chloroform were prepared and coated onto test tubes. The concentrations of Iodogen used were 1 µg, 5 µg, 7 µg, 8 µg, 9 µg, 10 µg, 30 µg and 50 µg per 20 µL of chloroform. Aliquots containing 100 µL MAb174H.64 (1.0 mg /mL) were added and incubated for 5 minutes in each of the coated test tubes. The immunoreactivity of the MAb174H.64 from each sample was determined in quadruplicate using the ELISA method of analysis as described in section 3.2.5.6.4. MAb174H.64, incubated in PBS was used as a positive control.

3.2.6.5. Effects of stannous ions on the stability of MAb174H.64.

Various concentrations of stannous chloride (1 μg, 5 μg, 10 μg, 20 μg and 50 μg in 250 μL of 1N HCl) were sealed in vials under nitrogen atmosphere. Aliquots of 100 μL of MAb174H.64 (1 mg/mL) in PBS were added to each vial. Then the preparations were incubated for 30 minutes, 6 hr and 24 hr, in triplicate. Aliquots from each sample was analysed by ELISA method, as described in section 3.2.5.6.4., to determine the immunoreactivity of the MAb174H.64 with the SCC cytokeratin protein.

3.2.7. Biodistribution of MAb-174H.64 in Different Animal Models.

3.2.7.1. Preparation of animal models.

The following SCC tumor cell lines suspended in PBS were obtained from Biomira Inc., Edmonton. These were KLN-205 cells (murine), FaDu cells (human) and SiHa cells (murine). Each of the Balb/c nude mice and/or DBA mice were injected subcutaneously, into the left flank, with $1x10^6$ cells per 0.1 mL. When the tumor grew to the desired size of 300-500 mg (8-10 mm) in about two to three weeks, the animals were used for biodistribution and whole-body scintigraphic studies.

3.2.7.2. Handling of tissue samples.

The various mouse animal models, bearing different SCC tumors, were injected intravenously via the tail vein with radiolabeled antibody for biodistribution studies. The mice were sacrificed by cardiac puncture exsanguination under carbon dioxide anesthesia. The heart, lung, liver, kidney, spleen, tumor, stomach, large intestine and small intestine, were collected as whole organs, while samples were taken from the upper hind leg muscle tissues and the skin. The entire tail was analyzed for radioactivity remaining at the injection site. All tissue samples were blotted on absorbent paper to remove any excess blood. The samples were then placed into small plastic vials and weighed wet. The remaining carcass was cut into two portions and placed into 20 ml plastic vials and weighed wet.

3.2.7.3. Biodistribution of ^{99m}Tc174H.64 in Balb/c nude mice bearing KLN-205 allograft.

Balb/c nude mice (n=5) each weighing about 20 g, bearing the KLN-205 allograft, were injected i.v. through a tail vein with a mixture of 10 μCi (370 kBq)/12 μg of ^{99m}Tc174H.64 and 6 μCi (222 kBq) /6μg of ^{125}I-MOPC-21 (control) in a total volume of 0.1 mL. The mice were sacrificed 24 hour after injection. Then organs and tissues were collected following the procedure described in section 3.2.7.2.

The radioactivity, in counts per minute (cpm) localized in each of the samples, was determined in a Beckman Gamma

8000 gamma scintillation counter using separate windows for the two radionuclides i.e. 15-80 keV window for ^{125}I and 100-200 keV window for ^{99m}Tc radioactivity. The radioactivity in cpm administered to each animal was determined by counting a known volume of diluted injected dose along with the tissue samples. The localization of radioactivity in each organ was expressed as percent injected dose per gram and tissue-to-blood ratio. In all of the calculations the cpm counted for background radioactivity were used. The spill-over radioactivity to the ^{125}I window from ^{99m}Tc radioactivity was corrected by modifying the 'Dual label spillover correction' program in the Gamma 8000 gamma counter for radioiodines.

3.2.7.4. Biodistribution of ^{125}I-174H.64 in DBA mice bearing KLN-205 allograft.

DBA mice (n=8) each weighing about 20 g, bearing the KLN-205 allograft, were injected i.v. with a mixture of 8 μCi (296 kBq) /6 μg ^{125}I-174H.64 and 6 μCi (222 kBq)/6μg ^{131}I-MOPC-21 (control) in a total volume of 0.1 mL. The mice were sacrificed at post injection times of 6 hr (n=2), 24 hr (n=3) and 48 hr (n=3). The radioactivity in the tumor tissues and the other organs were counted in the same way as described in sections 3.2.7.2. and 3.2.7.3. The spill-over radioactivity to the ^{125}I window from ^{131}I radioactivity was corrected by using 'Dual label spillover correction' program in the Gamma 8000 gamma counter. The percent injected dose per gram tissue for each organ and the tissue-to-blood ratio was determined.

3.2.7.5. Whole-body clearance of ^{125}I-174H.64 in DBA mice bearing a KLN-205 allograft.

The information obtained from the biodistribution study in DBA mice in section 3.2.7.4. was used to determine the whole-body clearance of ^{125}I-174H.64. The total radioactivity in the mice, at each post injection time periods of 6, 24 and 48 hours, was calculated using the data obtained from the biodistribution study. The result of this study, presented as % of the remaining whole body radioactivity in the mice at different time periods, was fitted to a model 7 PCNONLIN nonlinear estimation computer program to develop % ID/wholebody vs time curve. This curve was further analysed to determine the slopes defining it using the curve stripping method of the program. The PCNONLIN program used was from the UpJohn co. Kalamazoo, Michigan 49001, U.S.A. and modified to work on MTS at the University of Alberta.

3.2.8. WHOLE-BODY SCINTIGRAPHY.

3.2.8.1. Whole-body scintigraphy of DBA mice bearing a KLN-205 allograft.

Whole-body scintigraphy were performed on two DBA mice bearing KLN-205 allografts, one injected i.v. with 150 μCi (5.55 MBq)/200 μg of ^{125}I-174H.64 and the other injected i.v. with 100 μCi (3.7 MBq)/150 μg of ^{131}I MOPC-21 (control). Each mouse was anesthetized with an i.p. injection of 0.2 mL of a mixture of Ketamine and Xylazine, 10 minutes before imaging.

This anesthetic was prepared by mixing 0.5 mL of Ketamine solution (100 mg/mL) and 0.3 mL of Xylazine (20 mg/mL) in a final solution made to 6 mL with normal saline.

Imaging was performed at post injection times of 6 hr, 24 hr, 48 hr, and 120 hr using a gamma camera (Pho/ Gamma IV, Searle Instruments, Nuclear Chicago Corporation, U.S.A.) fitted with a 4 mm diameter aperture pin-hole collimator and attached to an ADAC (CAM III , ADAC lab., 540 Alder drive, Melpitas, Ca, U.S.A.) nuclear medicine computer system.

3.2.8.2. Whole-body scintigraphy of a Balb/c nude mouse bearing the FaDu xenograft.

A Balb/c nude mouse bearing a FaDu xenograft was injected i.v. with 900 μCi (33.3 MBq) /300 μg of ^{99m}Tc174H.64. The mouse was anesthetized prior to the imaging process, as described in section 3.2.8.1.

The whole-body scintigraphy was performed at 24 hr post injection time with a gamma camera (Pho/ Gamma IV, Searle Instruments, Nuclear Chicago Corporation, U.S.A.) fitted with low energy collimator. A computer (ADAC CAM III, ADAC lab., 540 Alder drive, Melpitas, Ca, U.S.A.) is interfaced with the gamma camera for collection and manipulation of the scintigraphy pictures.

3.2.9. The blood-level clearance of ^{131}I-174H.64 (IgG) and ^{125}I-174H.64 F(ab')$_2$ in normal rabbits.

New Zealand white healthy male rabbits (n=4) with an average body weight of 3.0 kg, were injected i.v. with a mixture of 300 μCi (11.1 MBq)/300 μg of ^{125}I-174H.64 F(ab')$_2$ and 250 μCi (9.2 MBq) /200 μg of ^{131}I-174H.64 (IgG) in a total volume of 200 μL. Samples of whole blood were collected with heparinized syringe from each of the rabbits, through their ear vein, at post-injection time periods of 0.08, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 13, 24, 36, 71 and 90 hours. The rabbits were anesthetized with 1 mL of Hypnorm (10 mg/mL of Fluanisone, Jonssen Pharmaceutical Ltd. Grove Oxford Ont.) and 0.5 mL of Diazepam (5 mg/mL, Sabex, Boucherville, Qc.) prior to the administration of radiolabeled MAb. As well as before each blood collection, the rabbits were anesthetized by i.m. injection of 0.25 mL of Hypnorm and 0.25 mL of Diazepam. The blood samples collected were analyzed by Beckman Gamma 8000 gamma scintillation counter. Two windows were set, one for ^{125}I (15 to 80 keV) and the other for ^{131}I (250 to 700 keV). The spill-over radioactivity to the ^{125}I window from ^{131}I radioactivity was corrected by using 'Dual label spillover correction' program in the Gamma 8000 gamma counter.

4. RESULTS AND DISCUSSIONS.

4.1 THE SCREENING OF TUMOR TISSUES AND CELL LINES.

The objectives of screening tumor tissues and cell lines on the bases of their immunoreactivity with MAb174H.64 are two fold. The first is to investigate if there is any correlation between the immunoreactivity of the tumor tissues or the cell lines with MAb174H.64 and the amount of cells which are of the squamous cell type present in that tissue. The other is to identify SCC tissues that exhibit strong immunoreactivity so that these may be used for the isolation of SCC antigen immunoreactive with MAb174H.64.

Two different approaches were used in the screening process. One is the immunohistological staining method of screening tumor tissues and cell lines as described in section 3.2.1.1. The other is the utilization of radiolabeled MAb174H.64 to determine its immunoreactivity with tumor cell lines as described in section 3.2.1.2. The results of both methods of screening are presented in the following sections.

4.1.1. Immunohistological Screening.

Tumor tissues and cell lines from different origin of human and bovine sources were analyzed immunohistologically, by Dr. G.S. Brzezinska of Biomira Inc., Edmonton. This helped to select those tissues and cell lines which are strongly immunoreactive with MAb174H.64. The control study was done using MOPC-21, in place of MAb174H.64. The intensity of staining is graded on a relative scale that shows strongest staining (90-100 %) as 4, stronger staining (75-90 %) as 3,

strong staining (50-75 %) as 2, weak staining (25-50 %) as 1 and no staining as 0. The percentage of positive cells indicates the percentage of cells positively stained for squamous cell typing.

The results of the screening process are presented as immunohistochemical staining of MAb174H.64 with frozen sections of different human tumor tissues and bovine squamous tumor tissues, in Tables 4 and 5 respectively. The results of the immunochemical staining of the monoclonal antibody with different human and a murine cancer cell line are presented in Table 6.

Table 4 shows the immunoreactivity of the different human tumor cells with MAb174H.64 and the percent of positive squamous cell type present in each tissue, determined by immunohistological methods. This helps to identify which tumor type is immunoreactive with MAb174H.64. The results show that out of 12 samples from different human adenocarcinoma tissues tested none has immunoreactivity with MAb174H.64. The same result was observed in sarcoma and lymphoma tissues tested. The tissue typing, to determine whether or not it is of squamous type, indicated that all of the adenocarcinoma, sarcoma and lymphoma tissues showed no squamous type of cells. The result of the immunoreactivity of the human squamous cell carcinoma (SCC) tissues from the different organs showed that out of the 60 samples all have strong immunoreactivity with MAb174H.64. Tissue typing analysis made

Table 4 MAb174H.64 immunohistochemical staining with frozen sections of different human tumors.+

Tissue samples	*No. positive/ No. tested	**Intensity of staining of tumor cells	***Immuno-reactivity unit (IRU)
Adenocarcinoma	0 / 12	0	–
–Ovarian Ca.	0 / 1	0	–
–Prostate Ca.	0 / 2	0	–
–Gastric Ca.	0 / 1	0	–
–Breast Ca.	0 / 5	0	–
–Colon Ca.	0 / 3	0	-
Sarcoma	0 / 1	0	–
Lymphoma	0 / 1	0	–
Squamous cell –carcinoma (SCC)	60 /60	4	400
–Lung Ca.	36 /36	4	400
–Head and neck Ca.	10 /10	4	400
–Uterine cervix Ca.	4 /4	4	400
–Skin Ca.	10 /10	4	400

+ This work was done by Dr. G. S. Brzezinska of Biomira Inc. Edmonton.

*Number of positively stained tissue samples per total analyzed.

**The intensity of staining was graded on the following basis: strongest = 4, weakest = 1 and no staining = 0. MOPC-21 was used as a control.

*** I.R.U., immuno reactivity unit is the multiplication product of the intensity of staining (0-->4 scale) and the percent of positive cells (0-100 %).

on the same samples indicated that all of the samples have 100% squamous cell type. The immunoreactivity unit (IRU), which is a measure of the product of the strength of immunoreactivity of MAb174H.64 with tumor tissues and the percent of cells in the tissue that are of squamous cell type, is presented in Table 4. The I.R.U. results show that all of the squamous cell carcinoma tissues analyzed have the maximum value of 400, while all of the adenocarcinoma, sarcoma and lymphoma tissue have zero IRU values. A control study was done with MOPC-21, a myeloma protein not immunoreactive with SCC tissues, replacing MAb174H.64. The result of the control study, not shown in the Table, indicated no immunoreactivity with any of the tumor tissues analyzed. This result in general shows that squamous cell carcinoma tissues are strongly immunoreactive with MAb174H.64, while adenocarcinoma, sarcoma and lymphoma tissues are not immunoreactive.

The results of immunohistological staining of MAb174H.64 with frozen sections of bovine squamous cell carcinoma tumor tissues are presented in Table 5. These results show that out of the 26 frozen tissue samples tested all had very strong immunoreactivity with MAb174H.64. The percent of squamous type of cells present in the 26 samples of the bovine tumor tissues show that 13 out of the 26 samples i.e. 50%, were 100% positive, 12 were 80% positive and one was 50% positive. The IRU values are 400 for the 13 samples, 320 for the 12 samples and 200 for the one sample. IRU values of 320 and

Table 5 MAb174H.64 immunohistological staining with frozen sections of bovine squamous cell tumors.+

Frozen tissue batch no.	Tumor site	*Intensity of staining of tumor cells	**Percent of positive tumor cells	***Immuno-reactivity unit (IRU)
T I	eyelid	4	100	400
T II	eyelid	4	100	400
T III	eyelid	4	100	400
T VI	eyelid	4	100	400
T V	eyelid	4	80	320
T VI	eyelid	4	100	400
T VII	eyelid	4	80	320
T VIII	eyelid	4	80	320
T IX	eyelid	4	80	320
T X	eyelid	4	80	320
T XI	eyelid	4	80	320
T XII	eyelid	4	80	320
T XIII	eyelid	3-4	80	240-320
T XIV	eyelid	4	80	320
T XVII	eyelid	4	100	400
T XVIII	eyelid	4	100	400
T XIX	eyelid	4	100	400
T XX	eyelid	4	100	400
T XXI	eyelid	4	100	400
T XXII	eyelid	4	100	400
T XXIII	mediastinum	4	50	200
T XXIV	eyelid	4	100	400
T XXV	eyelid	4	100	400
T XXVI	neck	4	80	320
T XXVII	lung	4	80	320
T XXVIII	eye lid	4	100	400

+ This work was done by Dr. G. S. Brzezinska of Biomira Inc. Edmonton.

*The intensity of staining is graded in relation of strongest to weak in 4 to 1 scale; Background staining using MOPC-21 showed no staining in all of the cases.

**Percent of Squamous type of cells in the tumor cell composition.

*** I.R.U., immuno reactivity unit represents, the multiplication product of the intensity of staining (0 —>4 scale) and the percent of positive cells (0 -100 %).

above are considered to indicate that the tumor is of squamous type of tissues strongly immunoreactive with MAb174H.64. Therefore out of the 26 bovine squamous cell tumor tissue samples tested 25 are strongly immunoreactive with MAb174H.64. These results again show that squamous cell carcinoma tissues are strongly immunoreactive with MAb174H.64. Two bovine tissue samples collected along with the tissue samples listed in Table 5, were from lung-mediastinum (batch number T XV and head and neck area batch number T XVI). Immunohistological analysis indicated that both samples are of non-squamous cell type and these showed no immunoreactivity with MAb174H.64. These results indicated that non-squamous cells are not immunoreactive with MAb174H.64.

Table 6 shows the immunoreactivity of MAb174H.64 with various human and one mouse cancer cell lines. MOPC-21 was used as a negative control. Out of the 16 human tumor cell lines analyzed 7 were of adenocarcinoma, 4 were of squamous carcinoma and the rest were of different non-squamous carcinoma types. The results show that one of the four human squamous carcinoma cell lines, SCaBER a bladder SCC, has 80% of the cell content to be of squamous cell type. This cell line showed an IRU of 320 that is an indication of a very strong immunoreactivity with MAb174H.64 and of high percent of squamous cell composition. The other three human tumor cell lines, FaDu, SKMES and SiHa cell lines showed little or no squamous type of cells in their cell composition and very

Table 6 MAb174H.64 immunohistological staining with different human and mouse cancer cell lines in culture+.

Cell Type	Tissue type	*Intensity of staining of cell	**Percent positive cells	***Immuno-reactivity unit (IRU)
FaDu	Pharynx squamous cell carcinoma	0	0	0
LS174T	Colon-adenocarcinoma	0	0	0
CaOV3	Ovary-adenocarcinoma	0	0	0
PC-3	Prostate adenocarcinoma	1	50	50
Du145	Prostate carcinoma metastasis in brain	0	0	0
COLO-205	Colon adenocarcinoma	0	0	0
MCF-7	Breast adenocarcinoma	1	50	50
C33A	Uterine cervix carcinoma	0	0	0
SKMES	Lung squamous cell carcinoma	1	10	10
SiHa	Uterine cervix (SCC)	0	0	0
647-V	Bladder carcinoma	0	0	0
SCaBER	Bladder squamous cell carcinoma	4	80	320
SKLU-1	Lung poorly differentiated adenocarcinoma	0	0	0
HT-3	Uterine cervix carcinoma metasta -size in lymph node	1	5	5
LoVo	Colon adenocarcinoma	0	0	0
LNCaP	Prostate carcinoma meta. in lymph node	0	0	0
KLN-205	Mouse SCC tumor	4	70	280

+ This work was done by Dr. G. S. Brzezinska of Biomira Inc.
*The intensity staining was graded on the following basis: strongest = 4, weakest = 1 and no staining = 0.
**Percent of squamous type of cells in the cell composition.
*** I.R.U., immuno reactivity unit is the multiplication product of the intensity of staining (0 —>4 scale) and the percent of positive cells (0 -100 %).

weak or no immunoreactivity with MAb174H.64. The mouse SCC cell line, KLN-205, showed very strong immunoreactivity with MAb174H.64 along with very high percent squamous type of cell composition.

The immunohistochemical staining results of the seven adenocarcinoma cell lines analyzed showed that five cell lines have no immunoreactivity with MAb174H.64. The remaining two adenocarcinoma cell lines, PC-3 (prostate adenocarcinoma) and MCF-7 (breast adenocarcinoma) showed weak immunoreactivity with MAb174H.64 and have 50% of their cell composition of squamous cell type. The presence of squamous cell type in adenocarcinoma cells in culture have been reported by other researchers as a possibility of co-existence of the adeno and squamous cell types (Quak J.J., 1989a).

Generally, all of the squamous carcinoma cell lines in culture with the exception of SCaBER and KLN-205 did not show strong immunoreactivity with MAb174H.64. These cells seem to have lost their squamous cell characteristics, because they also did not show the expected high percentage of squamous type of cell composition. One of the reasons for this type of loss of the immunoreactivity of SCC cells in culture could be that the cells, in the process of rapid growth in culture, might have shut-off or reduced their cytoskeletal protein synthesis and became deficient or virtually devoid of cytokeratin filaments. Similar cases have been reported that cytokeratin proteins in cells of SCC change their composition

when grown in culture media and that they regain their original composition when the cells are allowed to grow in tissues (Connell N.D. and Rheinwald J.G., 1983).

4.1.2. Immunoreactivity of radiolabeled MAb174H.64 with different types of tumor cell lines.

The immunoreactivity of MAb174H.64 with squamous cell carcinoma cell lines was determined using radiolabeled MAb174H.64. The cell lines used were FaDu, KLN-205 and SiHa cell lines. The procedure of this assay is presented in detail in section 3.2.1.2. Briefly ^{99m}Tc174H.64 was mixed with different amounts of the three cell lines separately and incubated for one hour at room temperature (22^{o}C). The percent radioactivity bound to the cells was determined and the results are presented as the percent bound radioactivity to FaDu, KLN-205 and SiHa cell lines in Tables 7, 8 and 9 respectively. ^{99m}Tc B80.3 which is not immunoreactive with SCC cytokeratin protein is used as a negative control.

The results in Table 7 show that the total radioactivity bound to the FaDu cells which originate from human pharynx, is less than 2.5% and this result shows no change even when the cell concentration is increased from 2.5 x 10^5 to 3.0 x 10^6. These values also are not different from the negative control study as shown by the percent specifically bound. Therefore it can be concluded that FaDu tumor cells grown in culture are not immunoreactive with ^{99m}Tc174H.64. The results in this assay are consistent with the results observed in the immunohistological staining presented in section 4.1.1.

Table 7 Immunoreactivity of FaDu cells with radiolabeled monoclonal antibodies ^{99m}Tc174H.64 and ^{99m}Tc-B80.3 (control)*.

No. of cells (in million)	MAbs used	**Radioactivity (CPM) in the Cell pellets	Total	Total percent bound	†percent specific bound
0.25	174H.64	1072	75632	1.35	0.22
0.25	B80.3	806	70521	1.13	-
0.5	174H.64	754	78614	0.95	0
0.5	B80.3	874	70765	1.22	-
1.0	174H.64	879	78310	1.11	0
1.0	B80.3	882	70825	1.23	-
2.0	174H.64	1218	77450	1.55	0.11
2.0	B80.3	1032	70468	1.44	-
3.0	174H.64	1786	78608	2.22	1.07
3.0	B80.3	824	70825	1.15	-

*The study was done in duplicate.

**The radioactivity of the standard, which is equivalent to the amount of radioactivity used for each reaction conditions for ^{99m}Tc174H.64 = 82293 CPM and for ^{99m}Tc-B80.3 = 78056 CPM.

†The percent specific bound = percent total bound ^{99m}Tc174H.64 minus percent total bound ^{99m}Tc-B80.3.

The KLN-205 cell line is a mouse squamous cell carcinoma. Table 8 shows the results of the immunoreactivity of the different concentrations of KLN-205 cells with ^{99m}Tc174H.64 and ^{99m}Tc-B80.3 (control). The results indicate that for all of the KLN-205 cell line samples with various cell concentrations, the total radioactivity bound to the cells is below 2%. The percent specific bound, which is the difference in the bound radioactivity between the ^{99m}Tc174H.64 and the control, is below one percent. These results indicate that the KLN-205 cells grown in culture showed no immunoreactive with MAb174H.64 when intact cell are used for the analysis.

Table 9 shows the results of the immunoreactivity of the SiHa cells with MAbs ^{99m}Tc174H.64 and ^{99m}Tc-B80.3 (control). SiHa cell line originated from human uterine cervix squamous cell carcinoma. The immunohistological test in the proceeding section showed that it has a weak immunoreactivity with MAb174H.64. The results in Table 9 show that the total percent radioactivity bound to the SiHa cells, for all of the different cell concentrations used, is below 3 percent which means that it is not immunoreactive with ^{99m}Tc174H.64.

The cell binding assay results for the FaDu and SiHa cells in culture are in confirmation with the immunohistology 4.1.1. However, the KLN-205 cell line which showed strong immunohistological reactivity failed to show any cell bound radioactivity in the ^{99m}Tc174H.64 cell binding assay. One possible reason for this could be that both FaDu and SiHa

Table 8 Immunoreactivity of KLN-205 Cells with radiolabeled monoclonal antibodies ^{99m}Tc174H.64 and ^{99m}Tc-B80.3 (control)*.

No. of cells (in million)	MAbs used	**Radioactivity (CPM) in the Cell pellets	Total	Total percent bound	†Percent specific bound
0.25	174H.64	1097	95927	1.13	0
0.25	B80.3	1554	111500	1.34	-
0.5	174H.64	1824	105720	1.70	0.68
0.5	B80.3	1174	113900	1.02	-
1.0	174H.64	1260	102439	1.23	0.09
1.0	B80.3	1102	96666	1.14	-
2.0	174H.64	1440	118038	1.22	0.16
2.0	B80.3	1210	114151	1.06	-
3.0	174H.64	1331	143118	0.93	0
3.0	B80.3	1210	80667	1.50	-

*The study was performed in duplicate.

**Radioactivity of standards, equivalent to the amount of radioactivity used for each reaction conditions for ^{99m}Tc174H.64 = 125622 CPM and for ^{99m}Tc-B80.3 = 153142 CPM.

†The percent specific bound = percent total bound ^{99m}Tc174H.64 minus percent total bound ^{99m}Tc-B80.3.

Table 9 Immunoreactivity of SiHa Cells with radiolabeled monoclonal antibodies ^{99m}Tc174H.64 and ^{99m}Tc-B80.3 (control)*.

No. of cells (in million)	MAbs used	**Radioactivity (CPM) in the Cell pellets	Total	Total percent bound	†percent specific bound
0.25	174H.64	1150	92081	1.23	0
0.25	B80.3	1474	82810	1.75	-
0.5	174H.64	1246	91739	1.34	0
0.5	B80.3	1340	83936	1.59	-
1.0	174H.64	1253	92254	1.34	0
1.0	B80.3	1432	82805	1.70	-
2.0	174H.64	2775	90659	2.79	0.64
2.0	B80.3	1811	84421	2.15	-
3.0	174H.64	1108	92001	1.19	0
3.0	B80.3	2154	81834	2.56	-

*The study was done in duplicate.

**Radioactivity of standards equivalent to the amount of radioactivity used for each reaction conditions. ^{99m}Tc174H.64 = 94808 CPM; ^{99m}Tc B80.3 = 86208.

†The percent specific bound = percent total bound ^{99m}Tc174H.64 minus percent total bound ^{99m}Tc-B80.3.

protein synthesis as a result of repeated growth in culture media. On the other hand the KLN-205 cells in culture might have slowed the cytokeratin filament production during the process of rapid growth in culture. In the immunohistological staining process of cells, the cytoplasmic content will be exposed as a result of cell lysis. Therefore in the case of the KLN-205 cells the cytokeratin protein thus exposed must have resulted in positive immunohistological staining. SCC cell lines grown in culture has been reported by other researchers to have lost their ability of synthesizing cytokeratin protein (Connell N.D. and Rheinwald J.G., 1983).

Cytokeratin proteins are intracellular components of squamous type of cells, radiating from the nucleus to the cell surface. In order for the MAb174H.64 (IgG) to interact with cytokeratin proteins the antibody has to penetrate the cell wall membrane or interact with cell surface component and become internalized (Matzku S. *et al.*, 1988). However IgG molecules are too large to pass freely through the cell wall membrane (Yuanfang L. and Chuanchu W., 1991) and may not have enough surface antigens to make any difference. These factors may explain why virtually no cell bound immunoreactivity was observed in all of the three SCC cell lines analysed.

4.2. IMMUNOREACTIVITY OF MAb174H.64 WITH CYTOKERATIN PROTEIN EXTRACTED FROM SCC TISSUES AND CELL LINES.

4.2.1. ELISA Results.

Cytokeratin proteins were extracted from different tumor tissues and tumor cell lines as described in section 3.2.2. The immunoreactivity of MAb174H.64 with cytokeratin protein extracted from the bovine SCC tissues, adenocarcinoma tissues, KLN-205 cell line and FaDu cell line were performed using the ELISA method of analysis as described in section 3.2.3.1. MAb170H.82, B80.3 and MOPC-21 were used as control antibodies.

The results of the ELISA analyses, presented in Table 10, show the degree of immunoreactivity of cytokeratin proteins extracted from bovine SCC tissues, adenocarcinoma tissues, KLN-205 cell line and FaDu cell line, with MAbs 174H.64, 170H.82, B80.3 and MOPC-21. As negative control study 6M guanidine HCl and phosphate buffered saline (PBS) were used.

Cytokeratin protein extracted from bovine SCC tissue of the eyelid region, as shown in Table 10, has very strong immunoreactivity with MAb174H.64. The optical density, which is a measure of the strength of the immunoreactivity, registered the highest intensity of +4. On the other hand cytoskeletal protein extracted from the adenocarcinoma tissue showed very weak reactivity with MAbs 174H.64 and B80.3 recording an intensity of +1 for each. However a stronger immunoreactivity was observed when the adenocarcinoma

extracted protein reacted with MAb170H.82. These results are in agreement with the results shown in Table 5, where bovine SCC tissues exhibited strong immunohistological reactivity with MAb174H.64.

KLN-205 and FaDu cell-extracted cytokeratin Protein Also showed strong immunoreactivity with MAb174H.64 as presented in Table 10. However, the immunoreactivity test result shown in Tables 7 and 8, indicated that both cell lines exhibited low or no immunoreactivity with MAb174H.64. Although the SCC cells in culture showed virtually no immunoreactivity with MAb174H.64, cytokeratin proteins extracted from the cells showed high immunoreactive because of the increased concentration of the protein in the extract.

Table 10 A typical ELISA analysis of the immunoreactivity of cytokeratin proteins from different sources of tumor tissues and cell lines with MAbs 174H.64, 170H.82, B80.3 and MOPC-21.†

Sources of cytoskeletal proteins	The degree of immunoreactivity in relative optical density *(OD).			
	174H.64	170H.82	B80.3	MOPC-21
Bovine SCC tissue (eyelid)	+4	0	0	0
KLN-205 SCC cell line (mouse tumor)	+4	+1	0	0
FaDu SCC cell line (human pharynx)	+3	+1	0	0
Adenocarcinoma tissue (breast)	+1	+3	+1	0
6M Guanidine HCl (control)	0	0	0	0
Phosphate buffered saline (control)	0	0	0	0

†Each test was done ten times per each microtiter plate of duplicate studies.

*The optical density read at 30 min. incubation time is presented as ranges of 0.95-1.3 as +4 (very strongly reactive); 0.7-0.95 as +3 (strongly reactive); 0.4-0.7 as +3 (weakly reactive); 0.2-0.4 as +1 (very weakly reactive) and less than 0.2 as 0 (non-reactive).

4.2.2. The Competitive Binding Assay.

In order to determine whether the immunoreactivity of MAb174H.64 with SCC cytokeratin protein is site specific or not, a competitive assay was performed as described in section 3.2.3.2. The results of the degree of inhibition of the various concentrations of MAb174H.64 on the binding of ^{99m}Tc174H.64 with SCC cytokeratin protein is presented in Table 11 and in Figure 11. MOPC-21 was used as a negative control.

Table 11 and Figure 11 show that increasing the concentration of non-radiolabeled MAb174H.64 increases the percent inhibition of binding of ^{99m}Tc174H.64 with SCC cytokeratin protein. The results are presented as percent inhibition of the amount of ^{99m}Tc174H.64 bound when different concentrations of the amount of unlabeled MAb174H.64 were added. The percent inhibition increased initially at a faster rate until 70% inhibition was reached at a ratio of 200 to 1 w/w of unlabeled to labeled MAb174H.64. Further addition of the unlabeled antibody added only slight changes in the percent inhibition. At a concentration of unlabeled to labeled MAb174H.64 of 500 to 1 w/w ratio, the percent inhibition started to reach a plateau at 85% inhibition. Further increase in the concentration of the unlabeled antibody did not change the percent inhibition proportionally, indicating that saturation of all the reactive sites had probably occurred.

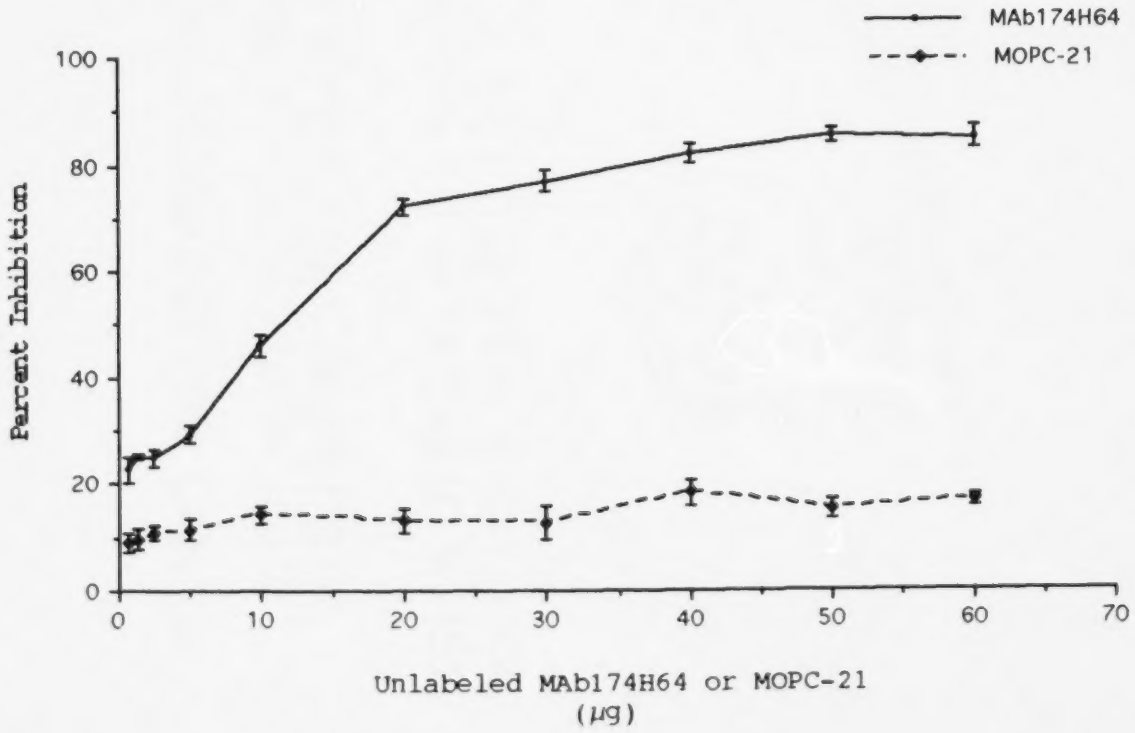
Table 11 Percent inhibition on the binding of ^{99m}Tc-174H.64* with SCC cytokeratin protein by unlabeled MAb174H.64 and MOPC-21 (control).

Amount of MAb174H.64 or MOPC-21 used in μg.	Percent Inhibition by** MAb174H.64	MOPC-21
0.63	22.7 (2.4)	8.8 (1.8)
1.25	25.1 (0.5)	9.6 (2.0)
2.50	25.0 (1.5)	10.8 (1.2)
5.00	29.3 (1.5)	11.4 (2.1)
10.00	46.0 (2.0)	14.2 (1.5)
20.00	52.5 (1.6)	13.0 (2.4)
40.00	66.4 (2.0)	12.6 (3.2)
50.00	84 4 (1.8)	18.2 (2.5)
60.00	85.3 (2.0)	16.9 (1.2)

*1.5 x 10^6 cpm /100 ng of ^{99m}Tc174H.64 was incubated in each of the microtiter well of the ELISA plate. At 0% inhibition the radioactivity was ≈560,000 cpm.

**The percent inhibitions are the mean values of three individual studies with the standard deviation in parenthesis.

Figure 11 Percent inhibition of the binding of 99mTc-174H64 with cytokeratin protein by unlabeled MAb174H64 and MOPC-21 (control). (n = 3, I = standard deviation)



This indicates that the non-radiolabeled MAb174H.64 and ^{99m}Tc174H.64 compete for the same epitope on the SCC cytokeratin protein structure. The control study performed using different concentrations of MOPC-21 to block the immunoreactivity of ^{99m}Tc174H.64 with the cytokeratin protein did not cause similar effects. Competitive ELISA has been described to be a useful method for detection of antibodies which react with one epitope (Kemeny D.M. and Chantler S., 1988). Hence, the result suggests that the reaction of MAb174H.64 with SCC cytokeratin protein is site specific. These results reinforced the ELISA observations reported in section 4.2.1. and confirm the specificity of the immunoreactivity of the MAb174H.64 with SCC cytokeratin protein.

Since the first monoclonal antibody against SCC was reported by Zenner H.P. and Hermann I.W. a number of other monoclonal antibodies were produced (Zenner H.P. and Hermann I.W., 1981). Most of these monoclonal antibodies were produced utilizing the whole SCC cell as the immunogen (Kyoizumi S., et al., 1985; Curry T.E., *et al.*, 1986; Kimmel K.A., *et al.*, 1986). As a result, most of the antibodies produced cross reacted with other tissue types such as adenocarcinoma, epithelial and non-epithelial tissues, and large cell carcinoma (Myoken Y., *et al.*, 1987; Quak J.J., 1989a, 1989b). Other monoclonal antibodies produced against non-SCC tumors but found to be immunoreactive with SCC were also reactive with other histological tumors and normal

tissues (Cowley G., *et al.*, 1986). These studies show that all of those previously reported monoclonal antibodies against SCC tumor lacked the specificity and non-cross reactivity required for a useful antibody. In comparison, the results from the immunohistological analyses, the ELISA assays and the competitive binding assays in this present study support that MAb174H.64 has a superior quality based on its specific immunoreactivity with SCC tumor.

4.3. CHARACTERIZATION OF CYTOKERATIN PROTEINS EXTRACTED FROM SCC TISSUE AND CELL LINES.

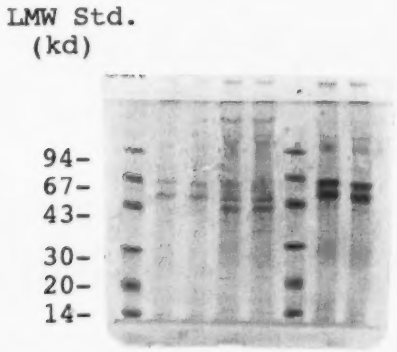
MAb174H.64 is shown to be strongly immunoreactive with squamous carcinoma tissues and some cell lines, as indicated in section 4.1. Additional results presented in section 4.2. revealed that cytokeratin protein extracted from SCC tissues and cell lines are specifically immunoreactive with MAb174H.64.

In view of these observations it became important to elucidate and characterize the cytokeratin protein which is immunoreactive with MAb174H.64. The composition of the SCC cytokeratin protein extract was studied using the SDS-PAGE electrophoresis method as described in section 3.2.2. The results of the SDS-PAGE electrophoresis of the cytokeratin proteins from bovine SCC tissues, KLN-205 cell lines and FaDu cell lines are presented in Figure 12. In the electrophoresis gel shown in Figure 12, the first and the 6^{th} lanes contain the low molecular weight standard proteins, the second and third lane contain KLN-205 cytokeratin, the fourth and fifth lanes contain FaDu cytokeratin and the last two lanes contain cytokeratin from bovine SCC tissues. The KLN-205 SCC cytokeratin protein showed two distinct bands lying between the 67 kd and 40 kd low molecular weight standard bands. The bovine SCC cytokeratin also showed two bands with high intensity at the same place as that of the KLN-205 cytokeratin bands. The cytokeratin protein extracted from FaDu cells showed a number of bands including the two bands

observed in the KLN-205 and the bovine SCC samples. This probably indicated that its cytokeratin protein composition is somewhat different to that of the KLN-205 cells and bovine SCC tissues.

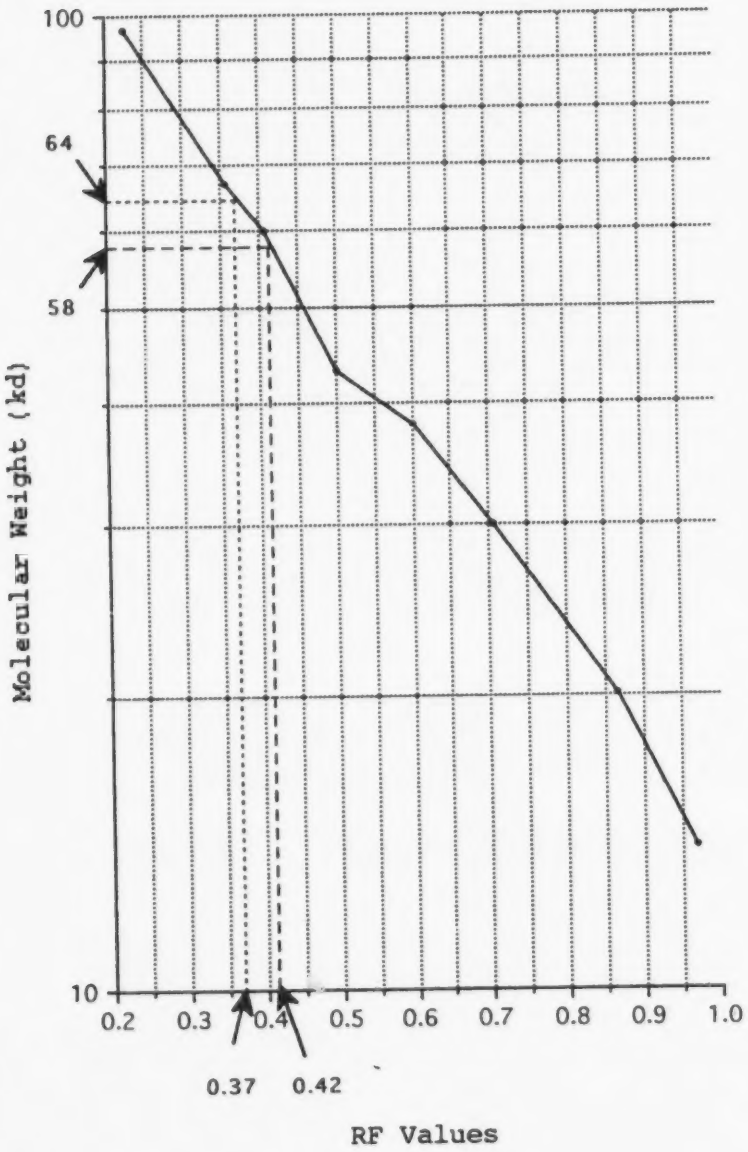
A molecular weight calibration curve was constructed utilizing high and low molecular weight calibration kits from Pharmacia® (S-7182, Uppsala 1, Sweden), as described in section 3.2.2. The standard curve, presented in Figure 13, was used to estimate the molecular weight of the cytokeratin proteins extracted from SCC tissues and cell lines.

Figure 12 SDS-PAGE with PhastGel Homogeneous 12.5, Coomassie blue stained.



The sample : LMW calibration protein lanes (1 and 6), KLN-205 cell extract (lanes 2 and 3)), FaDu cell extract (lanes 4 and 5), bovine SCC tissue extract (lanes 7 and 8). Total running time was 60 minutes.

Figure 13 Calibration curve established using the HMW and LMW calibration kits. (Pharmacia®) for SDS-PAGE on a 12.5% PAA gel.



The molecular weights of the two bands which appeared in all of the analyzed samples were extrapolated from the standard curve and were found to be 58 kd and 64 kd respectively.

Cytokeratin proteins are known to be expressed in a unique dimer units specific to the different types of epithelial cells. These dimer units are also known to be composed of an acidic (type I) and a basic (type II) sub units of polypeptides (Hatzfeld M. and Frank W.W., 1987, Hatzfeld M. and Weber K., 1990). The size of the acidic component of the dimer is smaller by about 8 kd than the basic component (Benz E.W., 1985).

On the basis of this classification, the two bands from squamous carcinoma tissues and cell lines, the 58 kd and 64 kd bands, constitute the acidic and the basic components of the cytokeratin protein respectively. The 58 kd murine acidic cytokeratin and its 56.5 kd human equivalent have been reported to be present in a number of SCC tumors classified as Moll's Catalogue #10 (Moll R.R. *et al.*, 1982; Quinlan A. *et al.*, 1989). The 64 kd murine basic cytokeratin and its 65.5 and/or 63 kd human equivalent are not commonly identified in squamous cell types but are classified as catalogue # 2 and/or 3 (Quinlan A. *et al.*, 1989). An attempt was made to separate and identify the cytokeratin components which are immunoreactive with MAb174H.64 using immunoblotting methods of the electrophoresis gel. However, it was not possible to get any positive results. One of the obstacles

was that the cytokeratin proteins for the electrophoresis procedure were dissolved in 9M urea. The presence of urea in the preparation most likely must have interfered with the immunoreaction between MAb174H.64 and the protein bands. There is no literature reported information found indicating the effect of urea interference in ELISA analysis. However, we have observed in a number of ELISA procedures that bovine SCC cytokeratin protein in 9M urea failed to show any immunoreactivity with MAb174H.64. This indicates that urea at high concentration likely interfered with the ELISA procedure.

The immunohistological analyses results of the various human and bovine tissues conclusively showed that MAb174H.64 is strongly immunoreactive with tumor tissues of squamous cell types. The analyses also showed that tissues of non-squamous cell type failed to immunoreact with the MAb. The control study using MOPC-21 showed no immunoreactivity with the SCC tissues. These results indicated that MAb174H.64 is selectively reactive with the human and bovine SCC tissues.

The immunohistochemical analyses on the various human and a mouse tumor cell lines grown in culture showed that those cell lines which retained their SCC cell type exhibited high immunoreactivity with MAb174H.64. It has been reported by researchers that SCC cell lines, on repeated growth in culture, looses their ability to synthesize cytokeratin protein (Connell N.D. and Rheinwald J.G., 1983). These results again confirm that MAb174H.64 is selectively

immunoreactive with SCC tissues.

The ELISA analyses on the cytokeratin proteins extracted from various bovine SCC tissues and SCC cell lines (FaDu and KLN-205) showed strong immunoreactivity with MAb174H.64 but not with MAbs 170H.82, B80.3 or MOPC-21. Cytoskeletal protein extracted from adenocarcinoma tissue showed no immunoreactivity with MAb174H.64 when ELISA was performed. The results of the competitive binding assays showed that the reaction of the MAb174H.64 with SCC cytokeratin protein is site specific. These results showed that the antigen responsible for the immunoreactivity of SCC tissues and cell lines with MAb174H.64 is the SCC cytokeratin proteins.

Electrophoresis analyses of the SCC cytokeratin protein extracted from SCC bovine tissue as well as from KLN-205 cell lines revealed a dimer unit composed of a 58 kd and 64 kd molecular weight cytokeratin proteins.

Analyses of the results obtained from the immunohistological studies, the ELISA and the competitive binding assays as well as the electrophoreses results strongly support that MAb174H.64 is a good marker for SCC tumor. Further study to isolate the antigens in a monomer form is recommended for a possible means of detecting circulating SCC antibodies in patients blood.

4.4. QUALITY CONTROL OF RADIOLABELED MAb174H.64.

Monoclonal antibodies including MAb174H.64 were labeled with ^{99m}Tc, ^{125}I and ^{131}I. The labeling procedures, methods of purification and the quality control of the final product have been described in section 3.2.5. Results for the radiochemical quality and the immunointegrity of radiolabeled antibodies are presented in the following paragraphs as section 4.4.1. and 4.4.2. respectively.

4.4.1. Radiochemical Quality Control.

Monoclonal antibodies were labeled with ^{99m}Tc by a direct labeling method as described in section 3.2.5.5. The radiolabeling yields and the radiochemical purity of the ^{99m}Tc labeled antibodies were determined by trichloroacetic acid (TCA) method, by instant thin layer chromatography analysis using different solvent systems, by HPLC, and by SDS-PAGE electrophoresis as described in sections 3.2.5.6.1., 3.2.5.6.2., 3.2.5.6.3 and 3.2.4. respectively.

The results of the radiolabeling yields of ^{99m}Tc labeled MAb174H.64, MAb-B80.3 and MOPC-21, determined by the trichloroacetic acid (TCA) method immediately after preparation, are presented in Table 12. The results show that the percent radiolabeling efficiencies for ^{99m}Tc labeled MAbs 174H.64, B80.3 and MOPC-21 are 95.2 % ± 2.1 , 92.4 % ± 2.6 and 92.1 % ± 2.2 respectively. The TCA method of analysis only reveals what fraction of the total radioactivity used is attached to protein and how much is in free form. It is not

Table 12 The TCA and ITLC (85% Methanol) method of analyses of radiolabeling yields of radiolabeled antibodies†.

Antibodies	Radionuclide	Radiolabeling yield*	
		TCA	ITLC
MAb174H.64	^{99m}Tc	95.2 ± 2.1	98.6 ± 1.2
MOPC-21	^{99m}Tc	92.4 ± 2.6	99.2 ± 0.2
MAb-B80.3	^{99m}Tc	92.1 ± 2.2	99.3 ± 0.3
MAb174H.64 (IgG)	^{125}I	94.0 ± 2.3	98.4 ± 1.1
MAb174H.64 [F(ab')$_2$]	^{125}I	91.2 ± 4.1	99.4 ± 0.6
MAb174H.64(IgG)	^{131}I	92.2 ± 3.6	98.6 ± 0.4
MOPC-21	^{125}I	92.9 ± 2.6	99.1 ± 0.6
MOPC-21	^{131}I	96.5 ± 1.5	99.2 ± 0.4

†Each of the radiolabeling conditions has been repeated at least 7 different times.

*The mean values, (± S.D.) of 4 determinations in one experiment

possible to discriminate among radiolabeled intact MAb, MAb fragments or MAb aggregates and colloids.

The results of the determination of the radiolabeling yield and radiochemical purity for ^{99m}Tc174H.64 using ITLC with different solvent systems, are presented in Table 13. The results show that with 85 % methanol as solvent, the labeling efficiencies were ≥ 98 %. The results obtained when 20% acetone in water or a mixture of 10 % ammonium sulfate: 20% methanol: water in the ratio of 2:4:4, were used as solvents were similar to that obtained when 85 % methanol was used. However, the chromatographic development time, when 85% methanol used as solvent, was only 6 minutes compared to more than 13 minutes for the other two solvent systems. Similar results were observed when ^{99m}Tc-B80.3 and ^{99m}Tc-MOPC-21 were analysed. The relatively short development time associated with the 85 % methanol makes it the solvent of choice. As with the TCA method the ITLC analysis was not useful to discriminate between the impurities due to the presence of radiocolloids, aggregated radiolabeled MAb and radiolabeled intact IgG or its' fragment.

Table 13 Comparison of the different solvent systems in the ITLC analyses of ^{99m}Tc174H.64 preparation.

Solvent system	*R_f values	% Radioactivity	Development Time
85% Methanol in water	0	99.2 ± 0.2**	6 minutes
	1	1.1 ± 0.2	
20% Acetone in water	0	98.6 ± 1.9	13 minutes
	1	2.1 ± 0.6	
†10% Ammonium sulfate and 20% methanol in water	0	98.3 ± 1.1	15 minutes
	1	1.8 ± 0.2	

* R_f = 0 represents the ^{99m}Tc174H.64, While R_f = 1 shows the $^{99m}TcO_4^-$ fraction

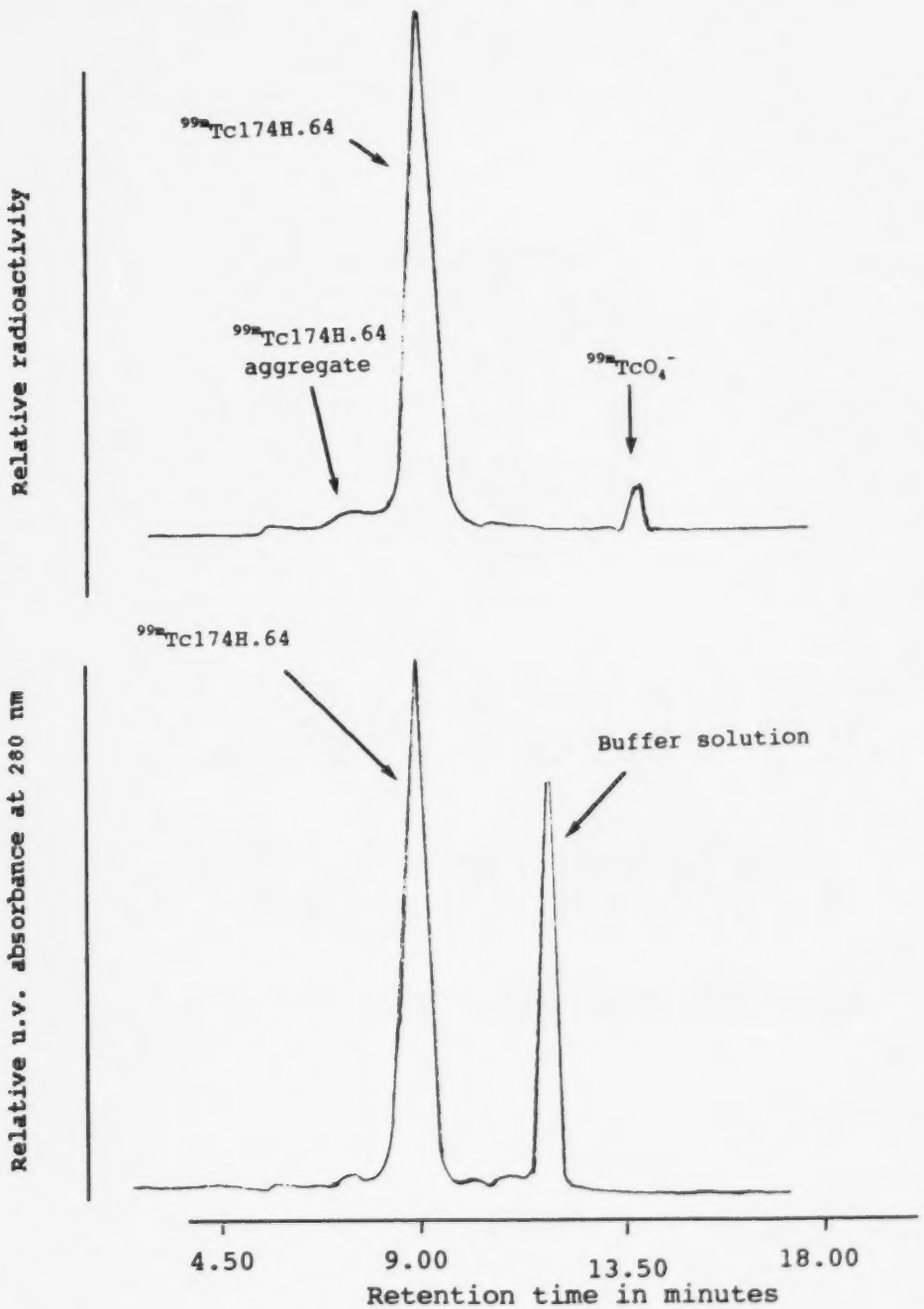
**Mean values with (± S.D.), of quadruplicate determinations.

†A mixture of 10% ammonium sulfate: 20% methanol: water in the ratio of 2:4:4

The radiochemical purity and radiolabeling yield of ^{99m}Tc labeled MAb174H.64 were determined using size exclusion HPLC method of analyses as described in section 3.2.5.6.3. The HPLC retention time for the uv absorbency was calibrated using MAb174H.64 and for the radioactivity peak using $Na^{99m}TcO_4$ and ^{99m}Tc174H.64. The calibration was performed in triplicate. The mean radioactivity retention times for $Na^{99m}TcO_4$ and ^{99m}Tc174H.64 were 14.2 ± 0.1 and 9.6 ± 0.1 minutes respectively. The mean retention time for the uv absorbency of MAb174H.64 was 9.6 ± 0.7 minutes. These retention time data were used as reference standards.

HPLC analyses were done on triplicate samples of ^{99m}Tc174H.64 preparations at 0, 6, and 24 hours post labeling periods. A typical HPLC analysis result is presented in Figure 14 as uv absorbency and radioactivity spectrum of the ^{99m}Tc174H.64 immediately after preparation. The uv spectrum shows two peaks, one at 9.8 and the other at 12.0 minutes retention time, representing the MAb174H.64 (IgG) and buffer component respectively. The radioactivity spectrum shows three peaks at, 8.2, 9.6 and 14.1 minute retention times. The radioactivity peak at 9.6 minute retention time is ^{99m}Tc174H.64, which is also detected as a uv absorbency peak at 9.8 minute. The 14.1 minute retention time peak represents $^{99m}TcO_4^-$. The radioactivity peak observed at 8.2 minute retention time probably corresponds to the small uv absorbency peak (shoulder) seen just before the 9.8 minute peak. This peak most probably represents high molecular

Figure 14 Typical uv absorbance spectra and radioactivity elution curve of the HPLC analysis of ^{99m}Tc174H.64 preparation.



weight aggregate of ^{99m}Tc174H.64, present as an impurity in a small fraction. A peak with similar absorbency retention time observed in monoclonal antibody analyses has also been reported by other groups although this fraction was not identified (Burchiel S.W., 1986).

The results of the HPLC analyses of the ^{99m}Tc174H.64 preparation at the different post-preparation time, is presented in Table 14. These results show a radiochemical impurity which was not detected by ITLC and TCA methods described earlier. The radiolabeled high molecular weight impurity was also detected in the uv spectrum as a small peak just before the main antibody peak. Since the size of the peak is relatively small, its retention time was not determined by the uv scanner. The fact that the peak appeared both in the uv and radioactivity spectra and that it appeared before the intact MAb suggests that the impurity is probably an aggregated ^{99m}Tc labeled MAb174H.64. The presence of radiolabeled IgG fragments, as possible impurities, were not identified by the HPLC analyses. The results in Table 14 also shows that the percent radioactivity for both the $^{99m}TcO_4^-$ and ^{99m}Tc-HMW-aggregate, increased with increased post-preparation time. The increase in the percent radioactivity was significant ($p < 0.025$). The increase in the percent of $^{99m}TcO_4^-$ and the radiolabeled HMW-aggregate, at 24 hr post-labeling time, were significantly more than the corresponding values observed in the 6 hr and 12 hr post-labeling periods ($p < 0.025$). Burchiel has also reported similar results

Table 15 HPLC analyses of the radiochemical purity of the ^{99m}Tc174H.64 at different post labeling time

Post labeling time		**Peak-Retention time in minutes		percent of applied radio-	Remarks
*No.	Hour	R.A. peak	uv peak.	activity	
1	0	8.2 (0.1)	-	4.1 (0.8)	HMW impurity†
2		9.6 (0.1)	9.7 (0.1)	94.3 (1.9)	^{99m}Tc174H.64
3		12.1 (1.8)	-	0.9 (0.4)	$^{99m}TcO_4^-$
1	6	8.0 (0.1)	-	4.0 (0.2)	HMW impurity
2		9.5 (0.1)	9.6 (0.1)	94.6 (0.1)	^{99m}Tc174H.64
3		14.3 (0.2)	-	1.4 (0.1)	$^{99m}TcO_4^-$
1	24	8.3 (0.1)	-	6.7 (0.4)	HMW impurity
2		9.7 (0.1)	9.8 (0.1)	81.3 (2.1)	^{99m}Tc174H.64
3		14.8 (0.1)	-	9.5 (0.3)	$^{99m}TcO_4^-$

*Radioactivity peak and uv peak number.

**Retention time in minutes (± SD) for radioactivity peak (R.A. peak.) and uv protein peak (uv peak).

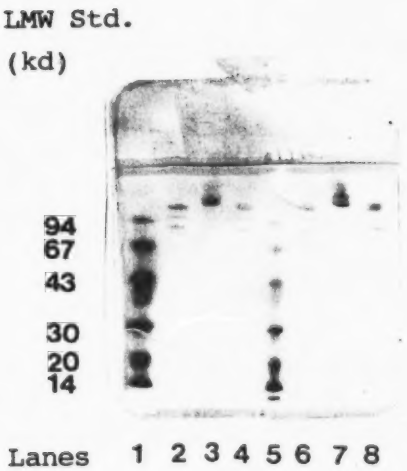
†HMW impurities may be polymers of the radiolabeled antibody.

(Burchiel S.W., 1986).

The SDS-PAGE electrophoresis method of analysis was performed to determine the presence of intact IgG, an impurity, in the preparation of radiolabeled $F(ab')_2$ fragment of MAb174H.64. This method of analysis used the non-denaturing condition which is described in section 3.2.2. Figure 15 shows a typical SDS-PAGE electrophoresis (12.5% homogenous gel) of the $F(ab')_2$ fragment of radiolabeled MAb174H.64 and the intact IgG. The Figure shows the intact IgG band at about 150 kd, the F(ab')2 band at about 100 kd, the heavy chain fragment band at about 50 kd and the light chains fragments band at about 25 kd. The intensity of the Coomassie blue is used to determine the relative concentrations of each components. This method would be more useful when used along with autoradiography.

The radiolabeling yields for ^{99m}Tc174H.64 determined by the HPLC analyses, immediately after labeling, was 94.3 % ± 1.9. The corresponding values determined by the TCA and ITLC methods were 95.2 % ± 2.1 and 99.2 %± 0.2 respectively. The high radiolabeling yields determined by the ITLC method obviously represent not only ^{99m}Tc174H.64 but also ^{99m}Tc174H.64-aggregate. The HPLC analyses however, not only determined the radiolabeling yield of the ^{99m}Tc174H.64 but also identified the radiochemical impurities present in the preparation. Therefore the size exclusion HPLC analysis is the method of choice for the determination of both the radiochemical quality and radiolabeling yields of

Figure 15 A typical SDS-PAGE electrophoresis (12.5% homogenous gel) of the $F(ab')_2$ fragment of MAb174H.64 (IgG) preparation.



The sample : LMW standard (lanes 1 and 5), MAb174H.64 $F(ab')_2$ fragment (lanes 2, 4, 6 and 8) and MAb174H.64 (IgG) (lanes 3 and 7). Total running time was 60 minutes.

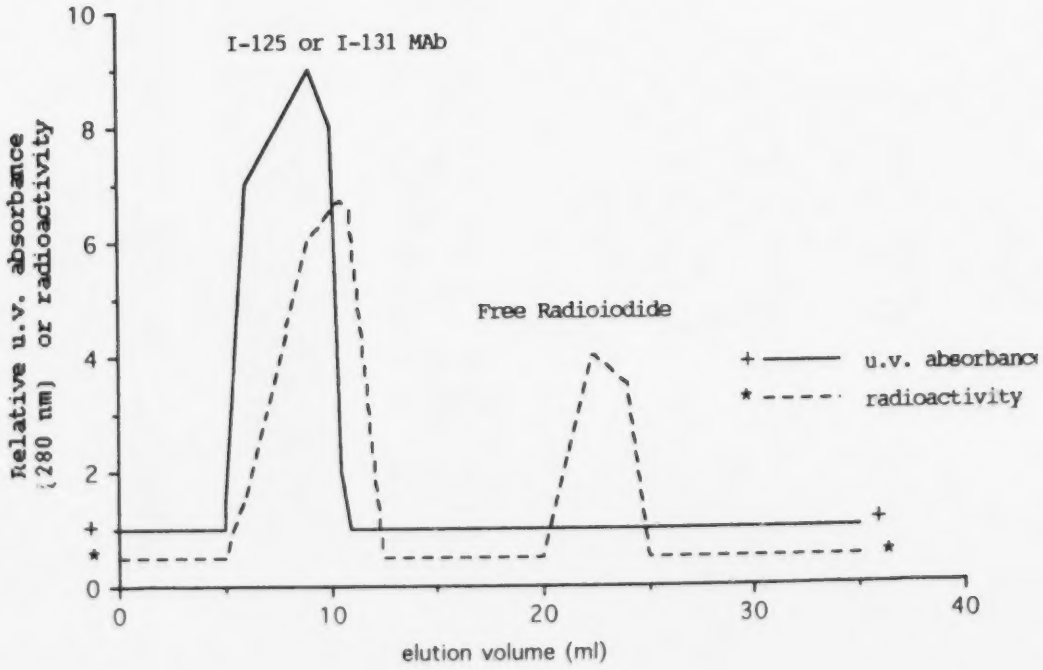
radiolabeled monoclonal antibody preparations.

Monoclonal antibodies were radioiodinated with ^{131}I or ^{125}I using the iodogen method of labeling, as described in section 3.2.5.3. The radioiodinated monoclonal antibodies were purified from the reaction mixture using size exclusion chromatographic technique as described in section 3.2.5.4.

A typical chromatographic elution curve for the gel chromatographic purification of radioiodinated monoclonal antibody preparation is presented in Figure 16. The Figure shows the radioactivity curve superimposed on the relative uv absorbency curve. This type of chromatography spectrum helps to identify the fractions which contain the radiolabeled protein.

The radiolabeling yields for MAb174H.64 and MOPC-21 radioiodinated with ^{125}I or ^{131}I, determined by the ITLC (85 % methanol) analyses, are presented in Table 12. The results show that radiolabeling yields for ^{131}I and ^{125}I labeled MAb174H.64 (IgG) and ^{125}I and ^{131}I labeled MOPC-21 (IgG) were > 98 % and that for ^{125}I-MAb174H.64 [$F(ab')_2$] were > 99 %. The radiolabeling yields determined by the ITLC method using 85 % methanol as solvent were higher than that obtained by the TCA method. This could be due to the harsher reaction condition in the TCA method which may have resulted in the deiodination of proteins. In general both results show that the radioiodinated antibodies preparation exhibited very high labeling efficiencies. The ITLC analysis is a simple and fast method requiring smaller sample size for analysis. Therefore

Figure 16 P6DG Bio Gel® Chromatography elution profile of ^{125}I or ^{131}I-labeled monoclonal antibodies and free radioiodide.



it is a better method for determining radiolabeling yield, than the TCA method.

4.4.2. Immunoreactivity of Radiolabeled MAb174H.64.

The ELISA results on the immunoreactivity of the ^{125}I-174H.64, ^{131}I-174H.64 and ^{99m}Tc174H.64 with SCC cytokeratin protein described in section 3.2.5.6.4. are presented in Table 16 as the mean optical densities of triplicate studies. The results show that all of the radiolabeled MAbs produce high optical density values similar to that of the unlabeled MAb174H.64. The results show no apparent reduction in immunoreactivity of the MAb174H.64 when specific activities of radioiodine of 5-10 μCi (185-370 kBq) /μg Protein And ^{99m}Tc of 14.5-27 μCi (0.5-1 MBq) /μg Protein Are used. Similar studies performed by Douillard J.Y., *et al.* indicated that radioiodinated MAb 17-1A, at specific activity of 4-10 μCi (148-370 kBq) /μg, produced strong immunoreactivity (Douillard J.Y., *et al.*, 1985). Specific activity in the range of 5-10 μCi (185-370 kBq)/μg has been shown by Goldenberg D.M., *et al.* to be equivalent to the binding of one atom of iodine per antibody molecule. A higher specific activity probably damages the immunoglobulin structure and may cause reduced immunoreactivity (Goldenberg D.M., *et al.*, 1980).

Table 15 Immunoreactivity of ^{131}I-174H.64 and ^{125}I-174H.64 with SCC cytokeratin protein.

Sample	Mean Optical Density*
^{131}I-174H.64	0.95 (0.02)
^{125}I-174H.64	1.04 (0.04)
^{99m}Tc174H.64	1.10 (0.05)
MAb174H.64 (control)	0.99 (0.02)
PBS (control)**	0.28 (0.04)

* Mean optical density of triplicate studies with standard deviation in parenthesis.

**Phosphate buffered saline (PBS) used as a negative control to determine the background optical density.

4.5. RADIOCHEMICAL STABILITY AND IMMUNOREACTIVITY OF RADIOLABELED MAb174H.64 EXPOSED TO VARIOUS PHYSICAL AND CHEMICAL CONDITIONS.

4.5.1. Radiochemical stability and Immunoreactivity of ^{99m}Tc174H.64 and ^{131}I-174H.64 incubated in Serum, Plasma and Urine *in vitro*.

Radiolabeled monoclonal antibodies residing in the body fluids are exposed to a number of physical and chemicals conditions. Transchelation of radionuclide bound to MAb, by sulfhydryl containing amino acids in blood is one such condition. The presence of sulfhydryl-containing proteins in blood, such as cysteine and glutathione have been reported to cause transchelation of ^{99m}Tc labeled to MAb (Hnatowich D.J., *et al.*, 1993; Cooper A.J.L., 1983; Lash L.H. and Jones D.P., 1985). The action of added human serum albumin to act as a free radical scavenger and to protect radioiodinated MAb from degradation by autoradiolysis has been reported (Kishor R., et al., 1986; Zimmer A.M., *et al.*, 1987).

Plasma contains fibrinogen most of which is removed in the clotting process in the preparation of serum. Plasma rather than serum best simulates the *in vivo* condition, however the presence of anticlotting chemicals may cause interference in the experiment. This was the main reason why both fluids were used. The effect of incubation in the body fluids on the radiochemical stabilities and the immunointegrity of the radiolabeled MAbs was investigated.

The studies were performed, as described in section 3.2.6., to evaluate the radiochemical stability of both ^{99m}Tc174H.64 and ^{131}I-174H.64 incubated in various body fluids. The results of these studies are presented in sections 4.5.1.1. and 4.5.1.2. below. The labeling efficiencies of both ^{99m}Tc174H.64 and ^{131}I-174H.64 were determined, using the ITLC method of analysis with 85 % methanol in water as solvent, prior to the process of incubation. The results of these analyses showed that the labeling efficiencies for both ^{99m}Tc174H.64 and ^{131}I-174H.64 were > 99%.

4.5.1.1. Radiochemical stability of ^{99m}Tc174H.64 and ^{131}I-174H.64 incubated in serum, urine and plasma fluids.

The effect of the incubation on the radiochemical stability of the radiolabeled MAb174H.64 was evaluated. The study was performed as described in sections 3.2.6.1. and 3.2.6.2. respectively. Briefly, a known amount of the radiolabeled antibody was incubated in the various body fluids for a various periods of time. Aliquots of each preparation were then analysed using Protein A affinity chromatography to determine the percentage of intact radiolabeled MAb174H.64 in each of the samples. The results of these studies are presented in Tables 16 and 17, as mean values of the percent radioactivity associated with intact ^{99m}Tc174H.64 and ^{131}I-174H.64 respectively. The control study was performed using radiolabeled MAb174H.64 incubated in PBS.

Table 16 shows the results of Protein A chromatography analyses of ^{99m}Tc174H.64 incubated in the different body fluids for various periods. Protein A has affinity for the Fc portion of IgG class of antibody at a basic pH of 8.0. When the sample was eluted using a basic solvent, the intact radiolabeled monoclonal antibody was retained in the column, while all of the other chemicals including any free radioisotope and fragmented antibody were eluted. The radiolabeled antibody in the column was later recovered by eluting with an acidic solution. The radioactivity associated with the Protein A bound fraction represents the intact radiolabeled IgG.

Statistical analyses was done on the results in Table 16, using Student's *t*-test method. The results show that the mean percent bound fractions obtained, by incubating ^{99m}Tc174H.64 in serum, for 6, 12 24 and 48 hours exhibited no significant difference from that analyzed immediately after incubation ($P > 0.05$). The ITLC analyses, of the bound fractions in all of the samples, showed a mean value of 89.3% ± 1.4. This shows that the percent bound fraction for the serum incubated ^{99m}Tc174H.64 exhibit no significant variations due to length of time of incubation for upto 48 hours.

Table 16 The stability of ^{99m}Tc174H.64 incubated in various body fluids, analyzed by Protein A chromatography*.

Incubation time in hours	Percent bound fraction **			
	Serum	Plasma	Urine	Control***
0	70.0 (3.2)	73.3 (2.1)	71.5 (2.3)	72.3 (3.2)
6	72.4 (2.3)	72.0 (2.3)	69.8 (1.2)	71.5 (2.1)
12	66.3 (4.8)	68.6 (3.3)	68.6 (1.9)	68.9 (1.8)
24	68.9 (2.5)	69.2 (4.1)	68.0 (1.6)	66.6 (2.3)
48	69.4 (2.3)	73.9 (1.4)	70.6 (1.3)	70.5 (1.3)

*The mean percent recovery from the Protein A chromatography analyses was 82.1 ± 1.1.

**The percent bound fraction is the fraction of the total radioactivity which binds to the Protein A column, at basic pH, presented as mean values (± S.D.) of triplicate determination.

*** ^{99m}Tc174H.64 incubated in PBS was used as control.

Similar results were obtained when plasma or urine was used in place of serum. The control study was performed on ^{99m}Tc174H.64 incubated in PBS. for the various time periods used. No significant differences, in the percent bound fractions, were observed between the experiment and the control study ($p > 0.05$), for each of the corresponding time periods. The results indicate that incubation of ^{99m}Tc174H.64 in the different body fluids for up to 48 hours seems to cause no significant effect on the radiochemical stability of ^{99m}Tc174H.64. Very high radiochemical stability of ^{99m}Tc-antibody incubated in human serum albumin was reported by others (Garron J.Y., *et al.*, 1991). This has an important implication in clinical investigation since studies with ^{99m}Tc labeled MAb are conducted up to 48 hours post injection time.

The results of the Protein A analyses for ^{131}I-174H.64 incubated in the various body fluids are presented in Table 18. The results show the fraction of the total radioactivity representing the intact ^{131}I-174H.64, documented as the mean percent bound fraction at various incubation periods. At 6 hour post incubation time the serum incubated with ^{131}I-174H.64 showed that the mean percent bound fraction was 78.3% ± 2.7. ITLC (85 % methanol) analysis results of these fractions show > 98.0% labeling efficiencies. The changes in the incubation time did not cause significant difference in the percent bound fraction observed ($p > 0.05$) or the labeling efficiencies of the determined bound fractions ($p > 0.05$).

Table 17 Protein A chromatography* analyses of ^{131}I-174H.64 incubated in the various body fluids.

Incubation time in hours	Percent bound fraction.** Serum	Plasma	Urine	Control***
0	78.3 (2.7)	79.3 (2.9)	77.5 (2.7)	80.6 (3.1)
6	75.4 (2.4)	76.0 (3.3)	75.8 (0.6)	79.6 (2.2)
12	76.3 (2.0)	72.6 (2.3)	70.6 (1.9)	75.8 (1.9)
24	72.1 (2.5)	71.2 (1.6)	62.9 (1.8)	71.4 (3.2)
48	74.4 (3.3)	73.0 (4.0)	55.6 (2.8)	72.3 (2.1)

*The mean percent recovery from the Protein A chromatography analyses was 89.2 ± 1.3 %.

**The percent bound fraction is the fraction of the total radioactivity which bound to the Protein A column, presented as the mean values, (± S.D.) of triplicate determination.

***^{131}I-174H.64 incubated in PBS.

Similar results were obtained when plasma fluid was used in place of serum. The results of the Protein A bound fraction and their labeling efficiencies in the plasma and serum incubated ^{131}I-174H.64 were not significantly different from the results of the control studies ($p > 0.05$). Studies performed by other research groups on the stability of radioiodinated monoclonal antibodies incubated in human serum albumin and plasma (*in vitro*) showed similar results (Zimmer A.M., et al, 1987).

The results of incubating the radioiodinated MAb174H.64 in urine exhibited a decrease in the percentage of the bound fractions with increase in the incubation time. The variation in the percent bound fraction between the 0 minute (immediately) and 48 hours incubation time, shown in Table 17, was about 22%. The urine-incubated samples also showed that the percent bound fraction was significantly different from the result of the control studies, for the periods exceeding incubation time of 12 hours ($P < 0.05$). The results of the ITLC (85 % methanol) analyses on the bound fraction from the urine incubated ^{131}I-174H.64 showed > 98% IgG bound radioactivity at all of the incubation time periods. The loss of radiolabel from the ^{131}I-174H.64 incubated in urine, may be due to the effect of the different chemicals present in the urine. However, the cause of the deiodination process is not clear.

4.5.1.2. Immunoreactivity of ^{99m}Tc174H.64 and ^{131}I-174H.64 incubated in serum, urine and plasma fluids

The effect of incubation in the various body fluids, on the immunoreactivity of ^{99m}Tc174H.64 and ^{131}I-174H.64 with SCC cytokeratin protein, was determined using the ELISA method, as described in sections 3.2.6.1. and 3.2.6.2. The results of these studies are presented in Tables 18 and 19. Table 18 shows the degree of the immunoreactivity of ^{99m}Tc174H.64, incubated in the various body fluids, with SCC cytokeratin protein. The results are presented as optical densities of triplicate studies, to evaluate the effect of incubation for the different period of time. The results show that there is no significant variation observed in the immunoreactivity of the ^{99m}Tc174H.64 as a result of incubation in serum for the different time periods ($p > 0.05$). The difference between the results of the immunoreactivities of the serum incubated and the PBS incubated (control) ^{99m}Tc174H.64 were not significant ($p > 0.05$). Similar results were obtained in the case of plasma incubated ^{99m}Tc174H.64. The urine incubated radiolabeled antibody showed significantly lower degree of immunoreactivity compared to the PBS incubated (control) ^{99m}Tc174H.64 ($p < 0.05$). However, there is no significant difference in the immunoreactivity of ^{99m}Tc174H.64 due to variations in the incubation time in urine ($p > 0.05$).

Table 18 The immunoreactivity of ^{99m}Tc174H.64, incubated in the various body fluids.*

Incubation time in hour	Immunoreactivity of ^{99m}Tc174H.64 as the mean value of the Optical Densities**.		
	Serum	Plasma	Urine
0	0.94 (0.11)	1.03 (0.12)	0.75 (0.14)
6	0.86 (0.09)	0.95 (0.08)	0.68 (0.10)
12	0.91 (0.09)	0.91 (0.10)	0.66 (0.11)
24	0.97 (0.09)	1.03 (0.11)	0.70 (0.11)
48	0.89 (1.10)	0.99 (0.08)	0.64 (0.09)

*The negative control studies, performed using the various body fluids showed O.D. of 0.22 ± 0.11 (n=3). The positive control, with the PBS incubated ^{99m}Tc174H.64 showed a mean O.D of 0.96 ± 0.12 (n=3).

**Mean (SD), of triplicate determination.

The reduced immunoreactivity observed in the urine incubated ^{99m}Tc174H.64 may probably be due to the interference of urea, present in urine, with the process of the ELISA analysis. The ELISA interfering effect of urea has been noted in another procedure reported in section 4.3. Control studies were performed using ^{99m}Tc174H.64 incubated in PBS as a positive control and the body fluids alone as negative controls. The positive control showed mean optical density values(n=3) of 0.98 ± 0.04 and the negative controls showed 0.23 ± 0.01 (n=3).

The results of the triplicate studies on the stability of the immunoreactivity of ^{131}I-174H.64 incubated in the various body fluids are presented in Table 19. The results show the mean optical density as a measure of the immunoreactivity of ^{131}I-174H.64 with SCC cytokeratin protein. Control studies were performed using ^{131}I-174H.64 in PBS and the body fluids alone as positive and negative control respectively. The results show that ^{131}I-174H.64 produced an optical density of 0.96 ± 0.12, while all of the body fluids showed background immunoreactivity (0.32 ± 0.10). The results of the serum incubated ^{131}I-174H.64, in general, showed strong immunoreactivity with cytokeratin protein. The variation of the optical densities observed for the different incubation time periods was not significant ($p > 0.05$). The plasma incubated radioiodinated MAb174H.64 also showed similar results as those of the serum incubated samples. The intensities of the immunoreactivity of ^{131}I-174H.64 with SCC

Table 19 The immunoreactivity of ^{131}I-174H.64, incubated in the various body fluids, with SCC cytokeratin.

Time of incubation in hour	Immunoreactivity of ^{131}I-174H.64 as the mean value of the Optical Densities**.		
	Serum	Plasma	Urine
(5 minutes)	0.93 (0.09)	0.91 (0.10)	0.60 (0.11)
6	0.80 (0.11)	0.86 (0.08)	0.60 (0.11)
12	0.79 (0.10)	0.88 (0.07)	0.61 (0.11)
24	0.77 (0.10)	0.80 (0.11)	0.51 (0.11)
48	0.91 (0.10)	0.97 (0.06)	0.62 (0.10)

*The negative control studies, performed using the various body fluids showed O.D. of 0.32 ± 0.10 (n=3). The positive control, with the PBS incubated ^{131}I-174H.64 showed a mean O.D of 0.89 ± 0.05 (n=3).

**Mean (± SD), of triplicate determination.

cytokeratin protein, for both serum and plasma incubated samples, were similar to the positive control study. These results indicate that incubation of ^{131}I-174H.64 in serum or plasma fluid, for a period of up to 48 hours, did not seem to significantly affect its immunoreactivity with SCC cytokeratin protein ($p > 0.05$). These results show that the immunointegrity of ^{131}I-174H.64 in plasma or serum is stable for at least 48 hours. This suggests that the radiolabeled MAb will be clinically useful for at least 48 hours post injection time.

The results of the incubation of ^{131}I-174H.64 in urine showed significant reduction in the optical density observed ($p < 0.05$). However, variation in the incubation time did not show any significant change in the optical densities observed ($p > 0.05$). Therefore, there is enough evidence to show that incubation in urine has effect in the reduction of the immunoreactivity of the ^{131}I-174H.64 with SCC cytokeratin protein. The degree of reduction in the immunoreactivity is not affected by the length of the incubation time. Hence, the cause of these reduction may be attributed to the presence of urea in the urine.

4.5.2. Effects of Temperature on the Stability of MAb174H.64.

MAb174H.64 solution incubated for 30 minutes in a water bath at different temperature was analyzed for immunoreactivity with SCC cytokeratin protein using the ELISA method of analysis as described in section 3.2.6.3. The results of this study on the effect of heat on the stability

of the immunoreactivity of MAb174H.64 with SCC cytokeratin protein is presented in Table 20. The results in Table 20 show that incubation of the monoclonal antibody at temperatures of 22^{o}C and 37^{o}C for up to 60 minutes did not significantly affect the immunoreactivity of the monoclonal antibody ($p > 0.05$). Incubation at 50^{o}C for 5 minutes also showed no significant effect on the immunoreactivity of the monoclonal antibody ($p > 0.05$). However increasing the incubation time to 30 minute and 1 hour resulted in the significant reduction of the optical density ($p < 0.05$). A further increase in the incubation temperature to 60^{o}C resulted in more reduction of the immunoreactivity of the monoclonal antibody. The reduction in the immunoreactivity of the antibody increased with increasing temperature and incubation time. At 70^{o}C and an incubation time of 30 minutes the optical density reached that of the background. The monoclonal antibody formed a precipitate at about 100^{o}C due to denaturation of protein. This study indicates that incubating at temperature of 50^{o}C or more negatively affects the immunoreactivity of MAb174H.64. This effect is directly proportional to the time of incubation. While the high temperature used in this experiment were outside the scope of normal physiological conditions, this study, nevertheless indicated that the ELISA technique provided a sensitive indicator of monoclonal antibody immunoreactivity. MAb174H.64 incubated at 37^{o} C for a period of upto one hour showed no loss in immunoreactivity. This indicates that MAb174H.64

Table 20 Effect of heat on the immunological stability of MAb174H.64.

Incubation temperature	Immunoreactivity of MAb174H.64 as mean value of optical density. (Incubation time) 5 min.	30 min.	60 min.
22°C	0.86 (0.10)*	0.95 (0.07)	0.79 (0.11)
37°C	0.90 (0.09)	0.87 (0.08)	0.80 (0.10)
50°C	0.88 (0.12)	0.66 (0.11)	0.45 (0.09)
60°C	0.45 (0.10)	0.40 (0.10)	0.32 (0.08)
70°C	0.40 (0.09)	0.23 (0.09)	0.22 (0.07)
80°C	0.30 (0.08)	0.22 (0.07)	0.23 (0.07)
90°C	0.21 (0.09)	0.19 (0.02)	0.15 (0.10)
100°C	========== Precipitation*** ==========		

*Mean (± SD), n=4

**The background reactivity (optical density), determined using PBS instead of the antigen, was 0.21 (0.09).

*** At 100°C the antibody precipitated out of the solution.

maintains its' immunoreactivity at normal body temperature.

4.5.3. The Effect of Iodogen® on the Immunoreactivity of MAb174H.64.

The effect of varying the concentration of iodogen on the immunoreactivity of the MAb174H.64 was determined by ELISA methods, as described in section 3.2.6.4. The results of the study are presented in Table 21. The results show that MAb174H.64 incubated with iodogen at a concentration of up to 5 μg per reaction tube containing 100 μg of antibody is strongly immunoreactive with the cytokeratin protein. However increasing the concentration of iodogen to 6 μg per 100 μg of the MAb174H.64 showed a significant reduction in the immunoreactivity of the MAb174H.64 ($p < 0.05$). Further increase in the concentration of iodogen caused increased reduction in the immunoreactivity of the monoclonal antibody. Iodogen concentration above 7 μg per 100 μg of the MAb causes a total loss of immunoreactivity of the monoclonal antibody. Usually for radioiodination of proteins, Iodogen concentration ranging from 1 μg to 5 μg per 100 μg protein is used. It has been reported elsewhere that increasing the quantity of Iodogen to more than 10 μg /100 μg protein (transferrin) or increasing the time of exposure will alter the property of the protein (Regoeczi E., 1983). Therefore for optimum radiolabeling of proteins without compromising their immunoreactivity, the concentration of the Iodogen and the time of incubation have to be considered.

Table 21 The Effect on the immunointegrity of MAb174H.64 incubated with various concentration of Iodogen*.

Quantity of Iodogen in µg / 100 µg of MAb	Degree of immunoreactivity in mean OD_{280}**.
1.00	0.87 (0.04)
5.00	0.83 (0.06)
6.00	0.55 (0.10)
7.00	0.44 (0.06)
10.00	0.22 (0.04)
15.00	0.23 (0.03)

*The reaction time for each test was 5 min.

**Mean optical density (± SD) n=4.

4.5.4. The Effect of Stannous Chloride on the Immunointegrity of MAb174H.64.

Stannous chloride is used as reducing agent in the direct method of labeling monoclonal antibodies by ^{99m}Tc. The effect of the different concentration of stannous chloride on the immunointegrity of MAb174H.64 was studied as described in section 3.2.6.5. The results of this investigation are presented in Table 22. The results show the immunoreactivity of MAb174H.64 in mean optical density values for the different reaction time periods. Analysis of the results indicated that there is enough evidence to conclude that stannous chloride at various concentrations reaching a maximum of 50 μg per 100 μg of antibody does not cause any significant change in the O.D. of the radiolabeled MAb ($p > 0.05$). The changes in the incubation time did not show any significant difference in the immunoreactivity of the MAb174H.64 ($p > 0.05$). Therefore, this indicates that there is no significant effect detected on the immunoreactivity of ^{99m}Tc174H.64 due to the presence of stannous chloride up to a maximum quantity of 50 μg per 100 μg of MAb174H.64. The usual quantity of stannous ion used in the direct labeling of antibody with ^{99m}Tc varies based on whether the antibody was first reduced or not. Hence 2.5 μg of stannous ion per 100 μg antibody has been reported to be used to reduce both the antibody and the ^{99m}Tc (Pettit W.A., *et al.*, 1980). However if the antibody is already reduced, stannous ion of about 0.5 μg per 100 μg antibody has been used to radiolabel antibodies

Table 22 The effect of stannous ion on the immunoreactivity of MAb174H.64.

Amount of $SnCl_2$ in μg/100 μg†	Immunoreactivity* in optical density** at different incubation time.			
	5 min.	30 min.	6 hr.	24 hr.
1.00	0.93(0.02)	0.89(0.01)	0.88(0.01)	0.90(0.02)
5.00	0.89(0.09)	0.87(0.03)	0.90(0.10)	0.87(0.05)
10.00	0.96(0.02)	0.90(0.07)	0.87(0.07)	0.79(0.11)
20.00	0.82(0.01)	0.88(0.02)	0.80(0.10)	0.92(0.02)
50.00	0.91(0.09)	0.86(0.03)	0.79(0.12)	0.95(0.12)

*Immunoreactivity of MAb174H.64 with SCC cytokeratin protein.

** The mean optical density (± SD) of triplicate determination

† Stannous chloride per 100 μg MAb174H.64

with ^{99m}Tc (Schwarz A. and Steinstråber A., 1987). It has also been reported elsewhere that a stannous chloride concentration of 40 μg per 100 μg antibody was used in the direct labeling with ^{99m}Tc, without any apparent change in the immunointegrity of the antibody (Rhodes B.A., *et al.*, 1986). Therefore, these experiments have indicated that with the procedure used, with stannous chloride concentration of up to 50 μg/100μg antibody there is no effect on the immunoreactivity of the MAb174H.64.

4.6. THE BIODISTRIBUTION OF RADIOLABELED MAb174H.64 IN DIFFERENT ANIMAL MODELS.

4.6.1. Biodistribution of ^{99m}Tc174H.64 in Balb/c Nude Mice Bearing KLN-205 Allograft.

The primary aim of this study was to determine if radiolabeled MAb174H.64 would localize selectively in a SCC tumor. The study was performed on five Balb/c nude mice bearing SCC KLN-205 allograft and injected i.v. with a mixture of ^{99m}Tc174H.64 and ^{125}I-MOPC-21 (control). A 24 hour biodistribution study of 10 μCi (0.37 MBq)/12 μg of ^{99m}Tc174H.64 in the Balb/c nude mice were done as described in section 3.2.7.3. The ratio of the tissue-to-blood localization of the radiolabeled MAb174H.64 for the various organs at 24 hour post injection were calculated from the corresponding percent injected dose per gram of tissue. The tissue-to-blood ratio of radioactivity is a measure of the relative localization of radioactivity in each organ. The results are presented in Table 23 as the mean value of tissue-to-blood ratio for each of the various organs of the mice. A control study was performed in a similar way by co-injecting 6 μCi (0.22 MBq)/6 μg of ^{125}I-MOPC-21 along with the ^{99m}Tc174H.64. The results of the control study are also presented in the Table.

The results in Table 23 show that the mean tissue-to-blood ratio, of ^{99m}Tc174H.64 in the tumor is significantly greater than ($p < 0.025$) that of the other organs except for that of the liver and kidney. The results also show that the

Table 23 Biodistribution of ^{99m}Tc174H.64 and ^{125}I-MOPC-21 in Balb/c nude mice bearing KLN-205 allograft*.

Organ	Tissue-to-blood ratio** ^{99m}Tc174H.64	^{125}I-MOPC-21
Tumor	1.22 ± (0.28)	0.47 ± (0.12)
Spleen	0.49 ± (0.12)	0.21 ± (0.02)
Kidney	1.07 ± (0.18)	0.25 ± (0.02)
Liver	1.03 ± (0.21)	0.29 ± (0.03)
Lungs	0.47 ± (0.16)	0.44 ± (0.04)
Stomach	0.12 ± (0.01)	0.11 ± (0.02)
Colon	0.15 ± (0.06)	0.10 ± (0.01)
Muscle	0.17 ± (0.06)	0.11 ± (0.04)

* A 24 hour study of the mice injected i.v. with mixture of 0.37 MBq/12 μg ^{99m}Tc174H.64 and 0.22 MBq/6 μg ^{125}I-MOPC-21 (control).

**Percent injected dose per gram tissue, Mean ±(SD), n=5.
per gram blood

mean tumor-to-blood ratio of ^{99m}Tc174H.64 is significantly greater than that of the control study ($p < 0.025$). However, the tumor-to-blood ratio was not significantly greater than either kidney or liver-to-blood ratio ($p > 0.025$). The reason for the high radioactivity in the liver probably is due to the role of the reticuloendothelial system (RES) in the uptake of radiolabeled antibody (Nelp W.B., 1975; Nishi T., et al. 1981).

In the process of radiolabeling monoclonal antibodies with ^{99m}Tc using stannous chloride as a reducing agent, the formation of radiolabeled tin-colloid is a well documented fact (Petit W.A., *et al.*, 1978; Dugan M.A., *et al.*, 1973; Rhodes B.A., 1991; Eckelman W.C. and Steigman J., 1991). Colloidal particles are known to be easily picked up by kupffer cells of RES of the liver (Nelp W.B., 1975). Although the presence of ^{99m}Tc MAb-tin-colloid could not be determined in the ^{99m}Tc174H.64 preparation using the *in-vitro* quality control methods employed, the observed high radioactivity uptake by the liver is an indication of the presence of the colloid in the preparation. Kupffer cells are also capable of binding IgG molecules through surface receptors and this was reported to contribute to liver uptake of antibody from circulation (Nishi T., et al. 1981). As well, radiolabeled antibodies were shown to bind to hepatocytes and therefore contribute to the high liver uptake of radiolabeled antibody (Sancho J., *et al.*, 1984). The binding of antibody to Fc receptors located on cell membranes in the liver of most

experimental mouse animal models has been described to cause high uptake of antibodies by the liver (Keenan A.A., Harbert J.C. and Larson S.M., 1985; Buchegger F., *et al.*, 1983).

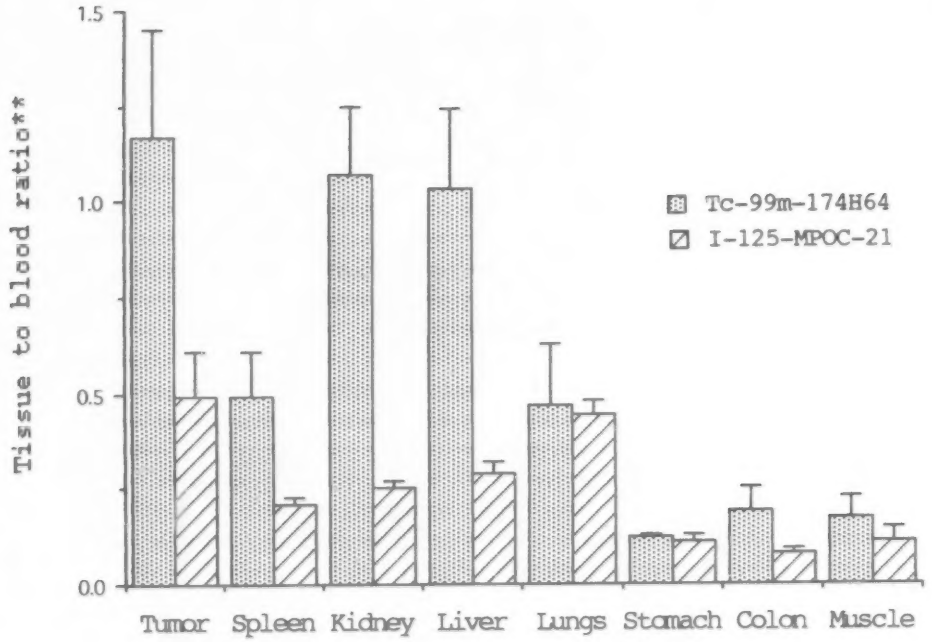
The high localization of ^{99m}Tc-radioactivity observed in the kidney is probably due to the transchelation of ^{99m}Tc by amino acid residue such as cysteine (Thakur M.L., *et al.*, 1991). One of the disadvantages of the direct labeling method using high concentration of reducing agent is the formation of the Fab' from F(ab')$_2$ as impurities (Zimmer A.M., *et al.*, 1987; Hnatowich D.J., 1990a). ^{99m}Tc-Fab' has been reported to be accumulated in the kidney of nude mice (Garron J.Y., *et al.*, 1991).

The over all information from these results indicate that at 24 hour post injection time, the tumor-to-blood ratio of the ^{99m}Tc174H.64 in the KLN-205 tumor is significantly more than that in the other organs except for the kidney and liver ($p < 0.05$). The KLN-205 tumor localization of the MAb174H.64 is also found to be significantly more than the localization of the control ^{125}I-MOPC-21 ($p < 0.05$). However this must be interpreted with caution since the metabolism of radioiodinated MAb differs from the metabolism of ^{99m}Tc labeled MAb.

Figure 17 shows the data in Table 23 in graphic form. Figure 17 shows that tissue-to-blood ratio of ^{99m}Tc174H.64 in the tumor is more than twice that for the other organs studied with the exception of the liver and kidney. The liver and kidney accumulation of the radioactivity have been

described to be mostly due to 1) the non-specific binding of antibodies to Fc receptors on hepatocytes; 2) antibody binding to circulating antigen and picked up by kupffer cells in the liver and 3) transchelation of ^{99m}Tc by cysteine residues which results in high kidney localization (Keenan A.A., Harbert J.C. and Larson S.M., 1985; Buchegger F., *et al.*, 1983; Thakur M.L., *et al.*, 1991). Consequently the high accumulation of ^{99m}Tc174H.64 in the KLN-205 tumor, shown in the results, indicated that the monoclonal antibody is strongly reactive to the SCC tumor.

Figure 17 A 24 hour biodistribution study of ^{99m}Tc174H.64 and MOPC-21 (control) in Balb/c nude mice bearing KLN-205 allograft*.



*Injected i.v. with a mixture of 0.37 MBq/12 μg ^{99m}Tc174H.64 and 0.22 MBq/6 μg ^{125}I-MOPC-21 (control).

**Mean ± SD (I), n=5.

4.6.2. Biodistribution of ^{125}I-174H.64 in DBA Mice Bearing a KLN-205 Allograft.

DBA mice bearing a KLN-205 allograft, were injected intravenously with mixture of 8 μCi (0.296 MBq)/6 μg ^{125}I-174H.64 and 6 μCi (0.222 MBq)/6 μg of ^{131}I-MOPC-21 (control). Biodistribution studies were performed at post injection times of 6 hr., 24 hr. and 48 hr. as described in section 3.2.7.4. The results of these studies are presented in Table 24 as the percent of injected dose per gram of tissue and tumor-to-tissue ratios. The 24 and 48 hours data in the Table represent the mean of triplicate studies, while the 6 hour values are averages of duplicate studies. These results are presented graphically in Figures 18, 19 and 20 as % I.D./g tissue at 6, 24 and 48 hours post injection time, respectively.

The results at 6 hr shown in Figure 18 indicated that the % I.D./g tumor tissue of ^{125}I-174H.64 was one of the lowest compared to the other organs, excluding that of the skin, colon and muscle. At 24 hr. post injection time the %I.D./g tumor tissue, shown in Figure 19, was more than most of the organs except for that of the blood and lungs. However at 48 hr. post injection time, shown in Figure 20, the % I.D./g tumor tissue was the highest compared to the accumulation in the other organs except in the blood.

Table 24 Biodistribution of ^{125}I-174H.64 following I.V. administration in DBA mice bearing KLN-205, tumor at various time intervals.

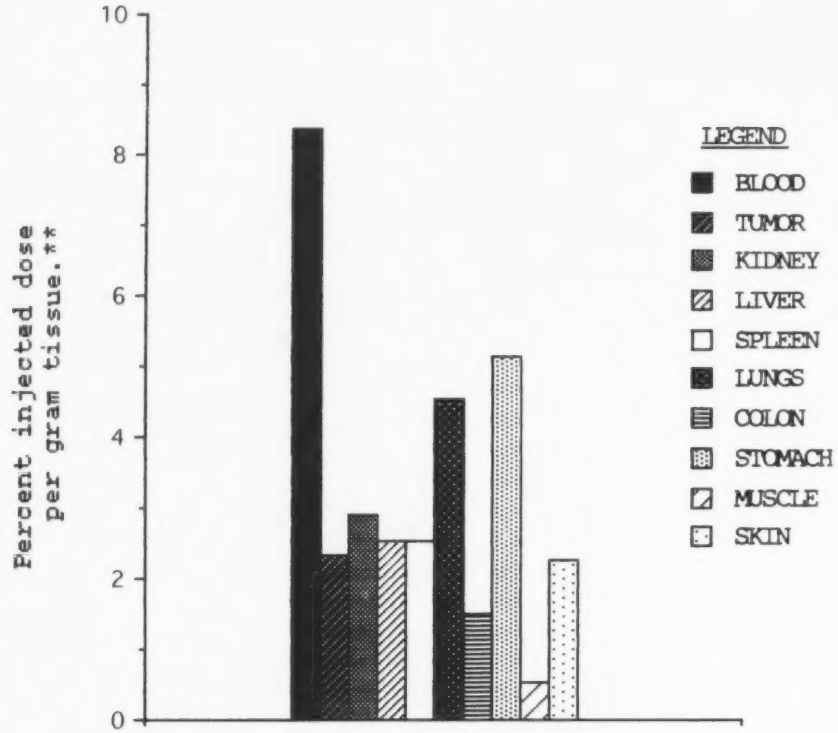
	Time (hours)		
Organ	6	24	48
Blood (a)	8.38 (7.98 & 8.78)	3.99 (1.31)	2.04 (0.84)
Tumor (a)	2.32 (2.31 & 2.33)	2.04 (0.12)	1.34 (0.16)
(b)	0.28 (0.29 & 0.27)	0.53 (0.15)	0.67 (0.06)
Kidney(a)	2.91 (2.86 & 2.96)	1.11 (0.61)	0.52 (0.16)
(b)	0.35 (0.36 & 0.34)	0.27 (0.02)	0.26 (0.03)
Liver (a)	2.53 (2.04 & 3.02)	1.18 (0.39)	0.56 (0.16)
(b)	0.3 (0.26 & 0.34)	0.30 (0.01)	0.28 (0.04)
Spleen(a)	2.52 (2.77 & 2.27)	1.03 (0.49)	0.47 (0.2)
(b)	0.3 (0.35 & 0.26)	0.30 (0.06)	0.23 (0.04)
Lungs (a)	4.55 (4.55 & 4.55)	2.19 (1.13)	0.96 (0.23)
(b)	0.54 (0.57 & 0.52)	0.53 (0.09)	0.49 (0.09)
Colon[1](a)	1.49 (1.43 & 1.55)	0.47 (0.17)	0.24 (0.09)
(b)	0.18 (0.18 & 0.18)	0.12 (0.01)	0.12 (0.01)
Stomach(a)	5.12 (6.23 & 4.01)	0.64 (0.16)	0.46 (0.11)
(b)	1.46 (1.91 & 1.0)	0.16 (0.01)	0.23 (0.04)
Muscle(a)	0.52 (0.68 & 0.36)	0.34 (0.16)	0.23 (0.08)
(b)	0.06 (0.09 & 0.04)	0.10 (0.07)	0.11 (0.13)
Skin (a)	2.26 (1.62 & 2.91)	0.91 (0.35)	0.77 (0.08)
(b)	0.3 (0.2 & 0.33)	0.25 (0.13)	0.43 (0.29)

(a) The mean % I.D./g tissue, ±(SD), n=3 and the average (range of values) from two DBA mice.

(b) The mean tissue-to-blood ratio, ±(SD), n=3 and the average (range of values) from two DBA mice.

[1] Small and large intestine with contents.

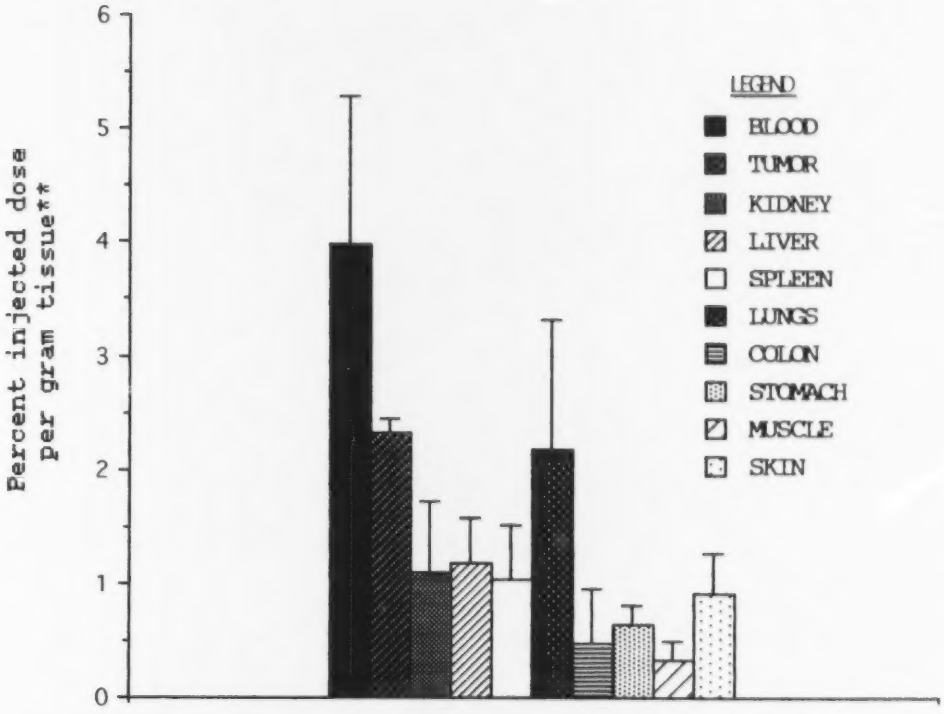
Figure 18 A 6 hr biodistribution of ^{125}I-174H.64 in DBA mice bearing KLN-205 allograft.*



* A 6 hr post injection study on DBA mice injected i.v. with 8 μCi (296 kBq)/6 μg of ^{125}I-174H.64

** Average value of duplicate studies.

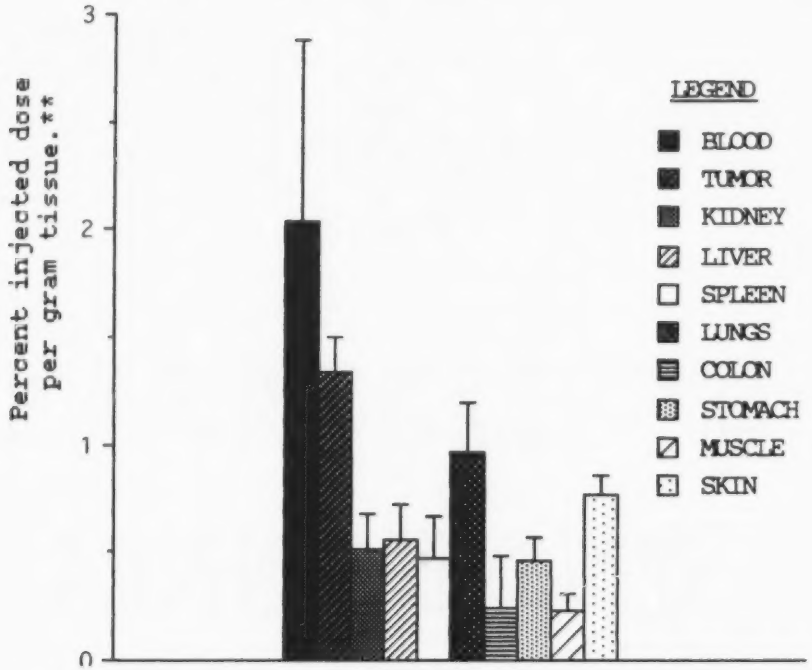
Figure 19 A 24 hr biodistribution of ^{125}I-174H.64 in DBA mice bearing KLN-205 allograft.*



* A 24 hr post injection study on DBA mice injected i.v. with 8 μCi (296 kBq)/6 μg of ^{125}I-174H.64

** Mean value, ± SD (I), n=3.

Figure 20 A 48 hr biodistribution of ^{125}I-174H.64 in DBA mice bearing KLN-205 allograft.*



* A 48 hr post injection study on DBA mice injected i.v. with 8 μCi (296 kBq)/6 μg of ^{125}I-174H.64

** Mean values, ± SD (I), n=3.

Statistical analysis of the 48 hr % I.D./g tissue in the various organs indicated that the localization of the ^{125}I-174H.64 in the tumor was significantly higher than in the other organs except that in the blood ($p < 0.025$).

The high accumulation of the relative radioactivity in the tumor is facilitated by the faster clearance of radioactivity from the blood and the other organs with time, compared to that of the tumor. The relatively fast clearance of the radioactivity in the organs other than tumor, helps in the reduction of background radiation and facilitates the tumor detection. The high uptake of radioactivity by the stomach at 6 hour period is likely due to the presence of free radioiodine or radioiodates. The main source of the free radioiodides is the dehalogenation process of the radioiodinated MAb174H.64 in the liver. Similar results have been reported by others (Carrasquillo J.A., *et al.*, 1987). A large amount of the free iodine accumulated in the thyroid gland because thyroid-block was not employed in this study.

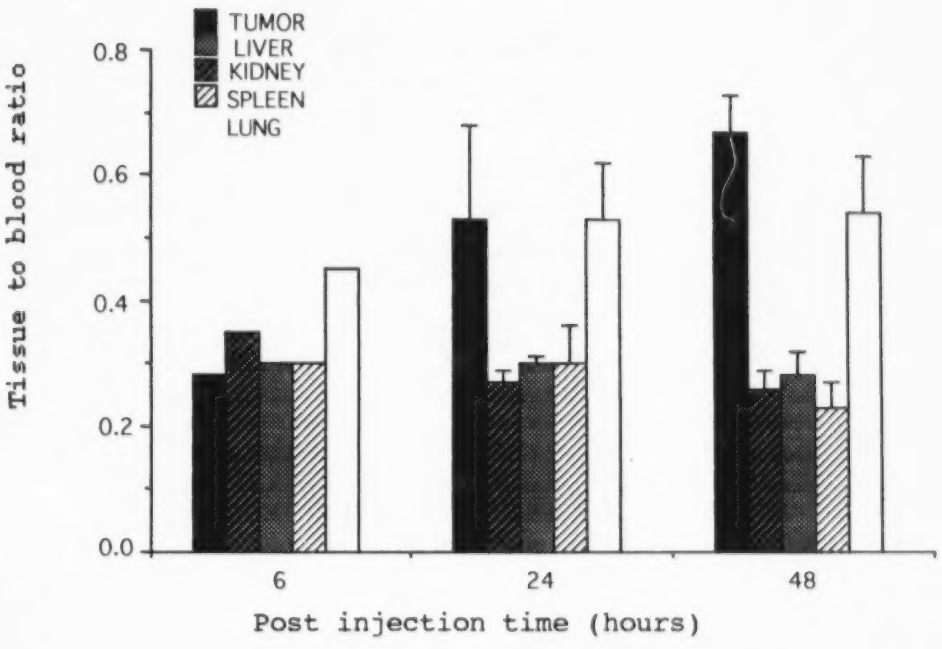
High uptake of radioactivity by the lungs and skin was observed. Lung and skin tissues both contain epithelial type of cells that contain cytoskeletal proteins of intermediate filament type (Blobel G.A., *et al.*, 1985; Samuel J. *et al.*, 1989; Fuchs E. and Green H., 1980). The immunoreactivity of MAb174H.64 with SCC tissue is based on its immunoreactivity with the intermediate filament cytokeratin protein present in the tissue. However, the high tissue-to-blood ratio observed

in the lung probably was because the lung contained a large amount of residual blood and that lung tissue is extremely light in weight.

The amount of radioactivity per gram tissue of each organ relative to that of the blood shows the pattern of effective localization in each organ. This is presented in Figure 21 as a ratio of the percent injected dose in the tissue of each organ to that in blood, at the various periods of time. The tumor-to-blood ratio at 6 hours is lower than the values for the other organs listed. However, at 48 hour the tumor-to-blood ratio exceeded all of the tissue-to-blood ratios of the other organs. The effect of all these events is a decrease in the background radioactivity while the relative tumor localization increases. The low background radiation effectively improves the detectability of the tumor mass in scintigraphy studies.

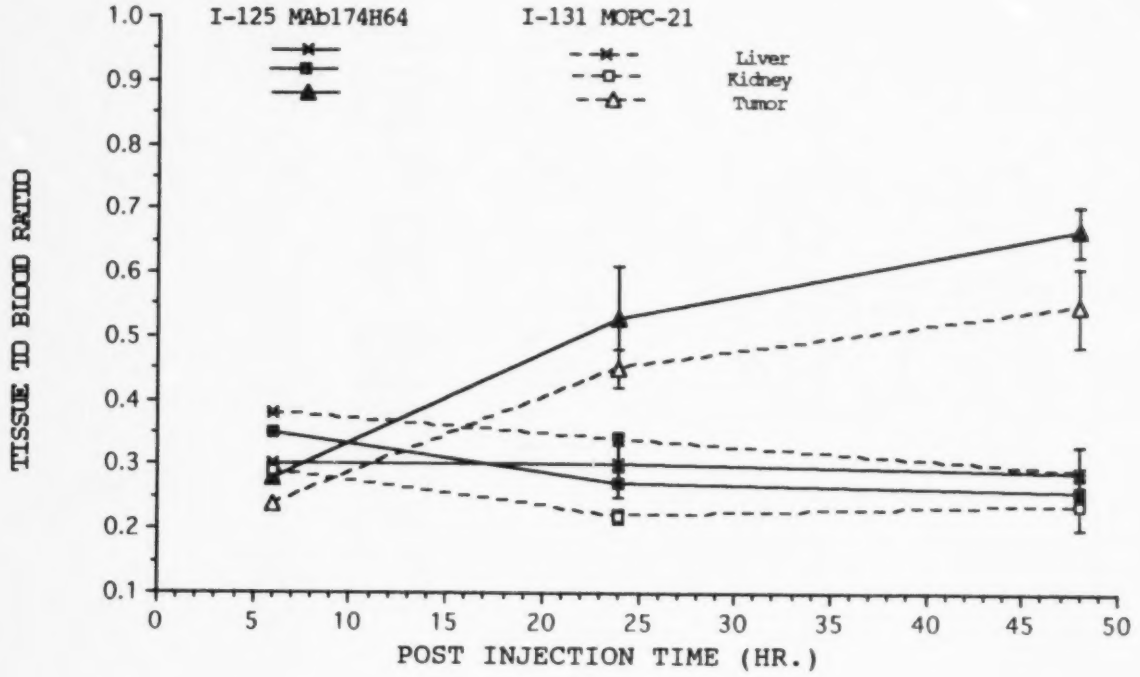
Figure 22 shows the biodistribution of the ^{125}I-174H.64 and ^{131}I-MOPC-21 (control) in DBA mice bearing a KLN-205 tumor. The results are presented as tissue-to-blood ratios at different post injection time. The Figure displays the tissue-to-blood ratios of the ^{125}I-174H.64 and ^{131}I-MOPC-21 (control) in tumor, kidney and liver. The results show that the ratio of both radioiodinated antibodies in the tumor increased with elapsed time, while in the kidney and liver the ratio decreased. At 6 hour the tumor-to-blood ratio was lower than the tissue-to-blood ratio of the kidney and liver, for both radioiodinated antibodies. This relationship becomes

Figure 21 Biodistribution (tissue-to-blood ratio) of ^{125}I-174H.64 in DBA mice bearing KLN-205 allograft.*



*The mice were injected i.v. with 296 kBq/6 μg ^{125}I-174H.64. The 6 hr data are averages of duplicates, while the 24 and 48 hours data are mean values, n=3 ±SD (I).

Figure 22 Biodistribution of 296 kBq/6μg of ^{125}I-174H64 and 222 kBq/6μg of ^{131}I-MOPC-21 (control) in DBA mice bearing a KLN tumor*.



*Mean values ± SD (I)

reversed at 24 hour, where the tumor-to-blood ratio becomes twice as much as the tissue-to-blood ratio of the kidney and the liver. Moreover, the biodistribution study at 48 hour shows that the tumor-to-blood ratios for ^{125}I-174H.64 is greater by a factor of 2.5 than the tissue-to-blood ratios for kidney and liver.

Comparison of the tumor-to-blood ratios for the ^{125}I-174H.64 and ^{131}I-MOPC-21 (control) shows a steady increase in the difference between the two values with elapsed time. At 24 hours and 48 hours post injection times the tumor-to-blood ratio of the ^{125}I-174H.64 was significantly higher than that of the control study ($p < 0.025$).

The biodistribution results presented in Figures 18 to 22 show that the relative localization of ^{125}I-174H.64 in the tumor increased with time. The localization of ^{125}I-174H.64 in the tumor is shown to be significantly higher than that observed in the control studies. Therefore, both of these results confirm that ^{125}I-174H.64 specifically localizes in the KLN-205 tumor grown in the DBA mice.

Comparison of the Balb/c nude mice and DBA mice used were made on the basis of tumor growth and cost of keeping the animals. Both were good animal models for growing the SCC KLN-205 tumor graft. Tumor growth in Balb/c nude mice was facilitated by their lack of a thymus gland which is likely responsible for immunorejection of any foreign graft. However, for the same reason they need more care in handling to prevent infection. This makes it more expensive to manage.

The DBA mice on the other hand do not easily allow tumor grafts, because of their intact immune system. However the KLN-205 tumor was first grown in DBA mice therefore there was no problem in growing the tumor in this animal model. The cost for keeping DBA mice is relatively smaller than the Balb/c nude mice.

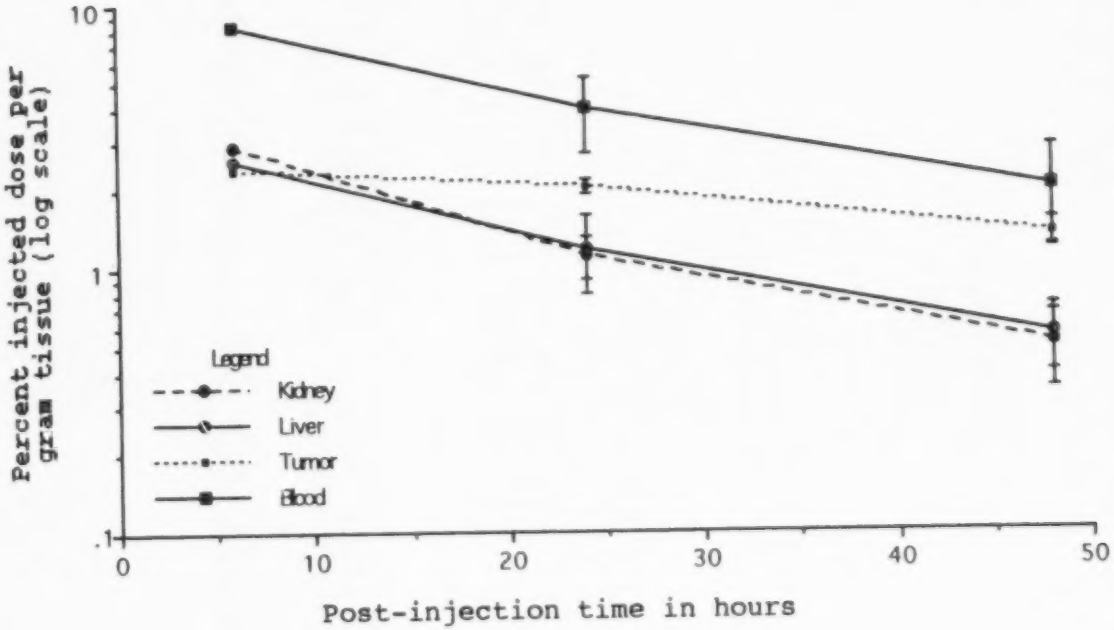
4.6.3. Clearance of ^{125}I-174H.64 from Critical Organs of DBA Mice Bearing A KLN-205 Allograft.

The data for the determination of the clearance of ^{125}I-174H.64 from the different organs of DBA mice is extracted from the results of the biodistribution study presented in Table 24, section 4.6.2. The clearance of radioactivity from blood, kidney, liver and the tumor at different post-injection times of ^{125}I-174H.64 in the DBA mice is presented in Figure 23. The clearance of ^{125}I-174H.64 from critical organs of DBA mice bearing KLN-205 allograft was analysed by a curve stripping method, although only 3 time points were available. This particular analysis indicated a biexponential clearance for each organ. Radioactivity from the blood circulation cleared primarily through a rapid rate with a half-life of 3 hours followed by a slower rate with a half life of 24 hours. The kidney showed a radioactivity clearance with half-lives of 5 and 24 hours for the fast and slow clearance respectively. The clearance of the radioactivity from the liver, spleen and lung showed similar rates as that of the kidney. The clearance of radioactivity from the tumor

indicated a relatively slower rate in relation to that of the other organs, with a half life of about 10 and 45 hours in each of the components.

The presence of high background radioactivity is a major obstacle in the technique of obtaining clear and diagnostically acceptable images. The major sources of background radiation in this case are radioactivity circulating in the intravascular and extravascular compartments and the non-specific radioactivity accumulation in tissues of different organs other than the tissue of interest, tumor. Therefore in scintigraphy studies it is very important to consider the time at which the target-to-background radioactivity is the maximum. Figure 23, shows that at a 6 hour post injection time the localization of radioactivity, expressed as a percent of the injected dose per gram of tissue, in the tumor was the smallest compared to the other organs analyzed. However because of the slower rate of clearance of the radioactivity from the tumor, the percent of the injected dose per gram of tissue, at 24 and 48 hours became significantly higher in the tumor, compared to that of the kidney and liver ($p < 0.025$). Therefore a post-injection time interval of 24 hr to 48 hr is recommended to obtaining a tumor scan of good quality.

Figure 23 Clearance of ^{125}I-174H64 (0.296 MBq /6 μg) from the critical organs of DBA mice (n=8) bearing KLN-205 allograft.*



*The 6 hr % ID/g tissues are average values (n=2), the 24 and 48 hrs are mean values of triplicate studies SD (I), n=3.

4.6.4. The Blood-Level Clearance of ^{131}I-174H.64 (IgG) and ^{125}I-174H.64 [F(ab')$_2$] Fragments in Normal Rabbits.

One of the problems encountered in the process of producing a good immunoscintigraphy of tumor is the high background radioactivity in the body. Often about 90% of injected radiolabeled antibodies remain in the background pool and only 1% or less localized in the tumor (Goodwin D.A., 1987). This high background radioactivity obscures the visualization of the localized radioactivity in the tumor mass. In order to overcome this condition it is important to identify the sources of the problem in detail.

There have been several factors identified which affect radiolabeled antibody background. These included the radiochemical purity of the radiolabeled antibody preparation, the affinity and specificity of the immunoreactivity of the antibody with tumor antigen and the degree of perfusion of the tumor. Considering the above variables at their optimum conditions, other important facts which can reduce the background radioactivity are currently undergoing investigation. These include: a) The use of antibody fragments, b) use of second antibody and c) use of a metabolic chelate linker (Yuanfang L. and Chuanchu W., 1991).

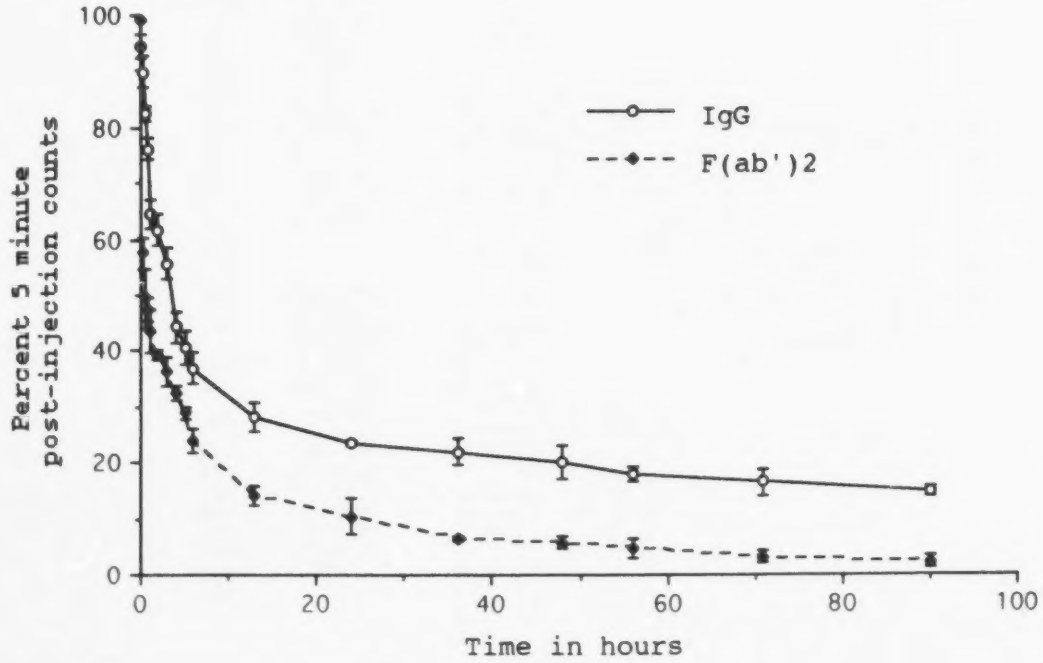
One promising approach is to replace the intact immunoglobulin with its fragmentation products. The intact IgG, because of its size has difficulty in diffusing out of the blood vessels. On the other hand the lower molecular size

fragments of IgG, the $F(ab')_2$ and Fab, clear faster from the blood and diffuse into the interstitial fluids to reach the tumor cells (Yuanfang L. and Chuanchu W, 1991).

The aim of this study was to determine and compare the rates of clearance of radioiodinated MAb174H.64 in the form of intact IgG and the $F(ab')_2$ fragment. The blood-level clearance of ^{131}I-174H.64 (IgG) and ^{125}I-174H.64 [$F(ab')_2$ fragment], from healthy rabbits (n=4) was investigated as described in section 3.2.9. Briefly, rabbits were injected intravenously through the ear vein with a mixture of 300 μCi (11.1 MBq)/300 μg of ^{125}I-$F(ab')_2$ and 250 μCi (9.2 MBq)/200 μg of ^{131}I-174H.64 (IgG). Blood samples were collected from the vein in the opposite ear at various post injection times and the mean radioactivities were determined. The mean radioactivity at the various periods were compared with that of the radioactivity at a 5 minute post-injection time. The results are presented in Figures 24 as a percent of the 5 minute radioactivity in blood for ^{125}I-174H.64 $F(ab')_2$ and ^{131}I-174H.64 (IgG).

Analysis of the blood clearance curve in Figure 24, by a stripping method using computer analysis revealed two components. The results show that the clearance of ^{125}I-174H.64 [$F(ab')_2$] from the blood is composed of a faster component with a half-life of 1 hour followed by a slower component with a half-life of 25 hours.

Figure 24 Blood clearance of ^{125}I-174H64 (IgG) and ^{131}I-174H64 [F(ab')$_2$ fragments] in rabbits*.



*Mean values, n=4, ± SD(I)

A similar analysis of the clearance of ^{131}I-174H.64 (IgG) from the rabbit blood indicated that there are two rates of clearance. The faster component has a half-life of 6 hours while the slower component shows a half-life of 58 hours. A study on the blood clearance of radioiodinated (^{125}I) anti-CEA whole-antibody and its $F(ab')_2$ fragments has been reported by Perkins and Pimm. The results showed that the whole-antibody cleared from the blood with a short half life (α phase) of 9.9 hours and a long half life (β phase) of 128 hours. The result also showed that the radioiodinated $F(ab')_2$ fragments have a short half-life of 2.95 hours and a long half-life of 7.9 hours (Perkins A.C. and Pimm M.V., 1991d). Patient blood-level clearance of ^{131}I-chimeric-MAb (mouse /human) has been reported elsewhere to be in the range of 17.6 ± 2.3 hours and 100.9 ± 16 hours for the short and long half-lives respectively (Meredith R.F., et al., 1991). Taking into consideration the difference in the type of MAb and the animal models used, the values obtained from both studies are in agreement with our findings.

Comparison of the rates of clearance of the radioiodinated intact IgG and its $F(ab')_2$ fragments of MAb174H.64 indicated that the fragmented antibody clears from the blood circulation at a faster rate than the intact IgG. The faster clearance of the $F(ab')_2$ fragment from the blood circulation is as a result of greater ease of extravasation into the intra cellular space because of its relatively small size. The intact IgG cleared from the blood circulation at a

slower rate because of its relatively larger size. Our result is in confirmation with many reported research works which show that $F(ab')_2$ and Fab fragments disappear much more rapidly from the blood than whole antibody (Yuanfang L. and Chuanchu W., 1991; Nelp W.P., *et al.*, 1985);

Radiolabeled MAbs in the blood must diffuse through the capillary walls and the interstitial fluid to reach the tumor cell. The whole-antibody (IgG) molecule, because of its large size, slowly crosses these barriers some times taking several days to attain a significant concentration for scintigraphy studies. However, radiolabeled MAb174H.64 $F(ab')_2$ fragments, due to their smaller size, easily diffuse through the blood capillaries and the interstitial space into the tumor.

The diffusion of antibodies from the blood compartment into the tumor is highly dependent on the concentration gradient between the blood and the tumor. The higher this value with time, the higher the tumor concentration will be. Although antibody fragments have a more rapid diffusion, their clearance from the blood is also more rapid. This will reduce the prolonged availability of the antibody in the system. Therefore, although the tumor-to-background radioactivity increases substantially, the actual amount of accumulated radioiodinated antibody in the tumor will be small.

On the other hand, radioiodinated MAb174H.64 (IgG) diffuses from the blood compartment at a relatively slow rate and causes a high background radiation due to the

radioactivity in the blood. Although the rate of extravasation is slow, because of the constant availability of the antibody in the blood, the amount of radiolabeled antibody in the tumor will be much more than that from the fragments (Goodwin D.A., 1987). However, because of the high background radioactivity, satisfactory images may not be attained. Reports comparing whole-antibody with its fragments showed high concentration gradients between the blood and the tumor to be more important than molecular weight, because they found higher tumor concentration with whole-antibody than its fragments (Wilbanks A.B., *et al.*, 1981; Wahl R.L., 1983).

In order to improve the detection of tumor it is very important to maximize the tumor-to-background ratio of radioactivity. Several approaches have been suggested to solve this problem, most of which are briefly presented in the literature survey of this thesis (Goodwin D.A. et al, 1986, 1987 and 1988).

In this experiment the observation that the intravenously injected radioiodinated $F(ab')_2$ fragment of MAb174H.64 in a rabbit animal model showed faster blood clearance than the radioiodinated whole-antibody was consistent with published data.

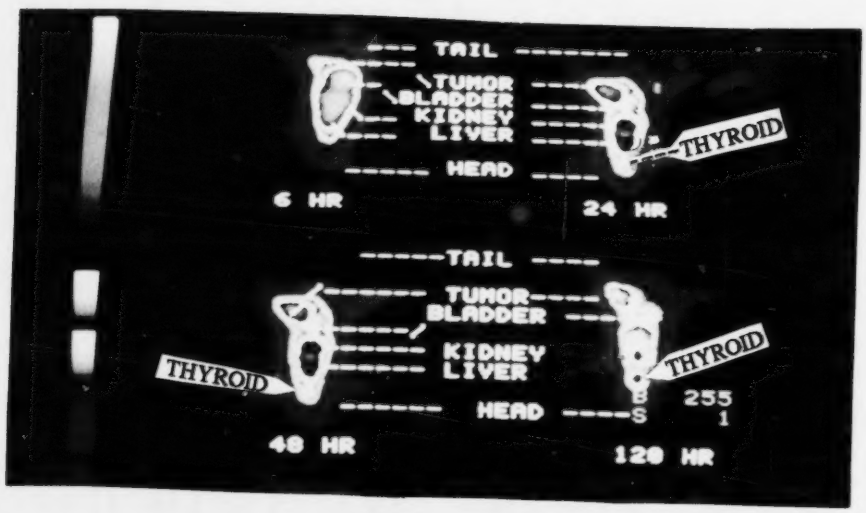
4.7. WHOLE BODY SCINTIGRAPHY.

4.7.1. Whole-Body Scintigraphy of DBA Mice Bearing a KLN-205 Allograft.

Whole-body scintigraphy was performed on two DBA mice bearing a KLN-205 allograft, as described earlier in section 3.2.8.1. Briefly, one of the mice was injected i.v with 150 μCi (5.55 MBq)/ 200 μg of ^{125}I-174H.64 and the other mouse was injected i.v. with 100 μCi (3.7 MBq) / 150 μg of ^{131}I-MOPC-21 (control). The scintigraphy results are presented in Figures 25 and 26. Figure 25 shows 6 hr, 24 hr, 48 hr and 120 hr post injection images of a KLN-205 tumor-bearing DBA mouse injected intravenously with the ^{125}I-174H.64. Figure 26 shows the same time period scintigraphy of another DBA mouse injected intravenously with ^{131}I-MOPC-21 as a control for non-specific localization.

The 6 hour scintigraphy of ^{125}I-174H.64 in Figure 25 shows that the whole-body radioactivity in the mouse is very high and as a result areas of radioactivity in the liver, kidney, bladder, stomach and tumor overlapped. Although a small protrusion extended to the left hind thigh muscle reveals the sight of the tumor, generally it is hard to separately identify the other organ localization. The 24 hour scintigraphy shows a reduction of the whole-body radioactivity by 50% and a distinct accumulation of the ^{125}I-174H.64 in the tumor. The accumulation of radioactivity is also detected in the thyroid, liver, stomach and kidneys. The radioactivities observed in the thyroid and the stomach are

Figure 25 Whole-body scintigraphy of a DBA mouse bearing a KLN-205 tumor, injected i.v. with 5.55 MBq / 200 μg of ^{125}I-174H.64.

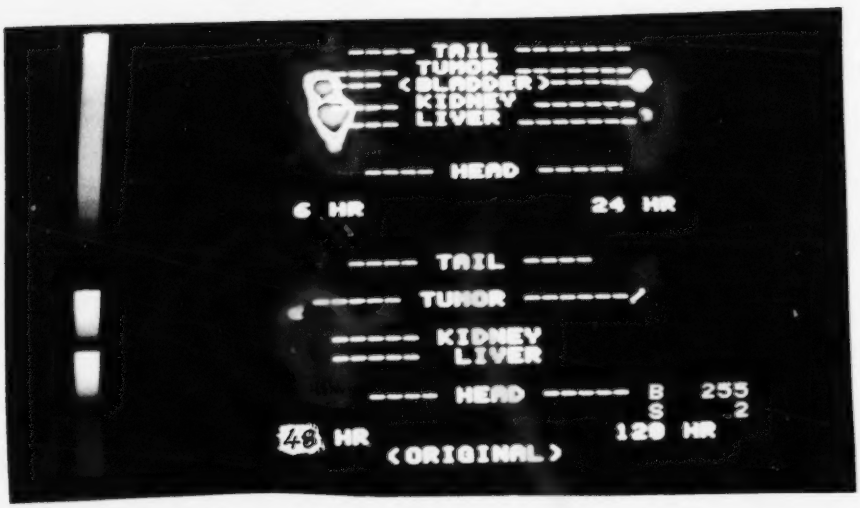


The whole body radioactivity per image for the 6, 24, 48 and 120 hours are 101500 counts in 10 minutes (10150 cpm), 99420 count in 20 minutes (4971 cpm), 79075 counts in 25 minutes (3163 cpm) and 29850 counts in 25 minutes (1194 cpm) respectively.

due to the free radio-iodine released from systemic deiodination of antibodies including catabolization in the tumor (Hnatowich D.J., 1989; 1990a). The accumulation of radioactivity in the liver is probably as a result of the binding of antibodies to Fc receptors located on liver cell membranes (Keenan A.M., *et al.*, 1985; Buchegger F., *et al.*, 1983). Degraded ^{125}I-174H.64 present in the preparation as Fab or F(ab)'$_2$ fragments usually get trapped in the kidney thereby increasing the non-specific localization of radiolabeled monoclonal antibody in the organ (Garron J.Y., *et al.*, 1991, Hnatowich D.J., 1990a). The 120 hour scintigraphy shows a 90% reduction of the 6 hour whole-body radioactivity. The same image shows that there is a distinct localization of radioactivity in the tumor, however thyroid, liver and stomach also showed appreciable accumulation of radioactivity. The DBA mice thyroid glands were not blocked by stable iodide prior to the administration of the radioiodinated antibodies.

A control scintigraphy study was performed with a KLN-205 tumor-bearing DBA mouse that was injected intravenously with ^{131}I-MOPC-21. The four time period whole body scintigraphy of this study is presented in Figure 26. The 6 hour image shows accumulation of radioactivity in the liver, kidney, stomach and the bladder. The 24 hour scintigraphy shows a considerable clearance of whole-body radioactivity with some radioactivity localized in the liver, kidney and bladder. Because of the proximity of the tumor to the bladder

Figure 26. Whole-body scintigraphy of a DBA mouse bearing a KLN-205 tumor, injected i.v. with 3.7 MBq/ 150 μg ^{131}I-MOPC-21.



The whole body radioactivity per image for the 6 hr, 24 hr, 48 hr and 120 hr are 94866 counts in 30 minutes (3162 cpm), 34843 count in 25 minutes (1393 cpm), 36876 counts in 45 minutes (819 cpm) and 10106 counts in 30 minutes (337 cpm) respectively.

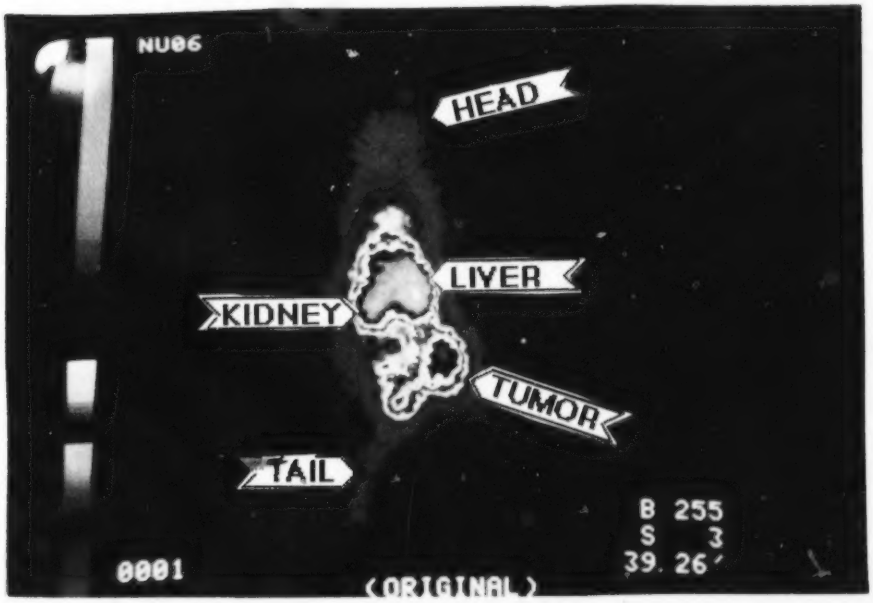
the radioactivity accumulated in the bladder would overlap and mask any localization of radioactivity in the tumor. To avoid this problem the 48 hour scan was taken in left lateral view. This image now shows that there is some localization of radioactivity in the tumor. The 120 hour study, a lateral view, also shows traces in the tumor localization of the radioactivity. The scintigraphy observed in Figure 25 clearly shows that ^{125}I-174H.64 localizes in the KLN-205 tumor of the DBA mouse. The 6 hr. whole-body radioactivity was reduced by a factor of 3 and 5 at 48 and 120 hours post injection times, respectively. The observation made in the control study shows non-specific tumor binding of the ^{131}I-MOPC-21. In the *in vitro* ELISA analyses reported in section 4.2.1. MOPC-21 has been shown to be non-immunoreactive with the SCC cytokeratin protein. Therefore the presence of MOPC-21 in the KLN-205 tumor is a non-specific localization. One way to avoid or minimize the non-specific localization may be to block the non-specific binding site (Taylor A., *et al.*, 1988; Carrasquillo J.A., *et al.*, 1988; Corhelius E., *et al.*, 1986). This can be achieved by injecting a non-immunoreactive MAb of the same IgG class prior to the injection of the sample. However, there is a possibility that low level specific binding sites could also be blocked resulting in diminished tumor uptake (Murray J.L., *et al.*, 1987).

4.7.2. Whole-Body Scintigraphy of a Balb/c Nude Mouse Bearing a FaDu Allograft, Injected Intravenously with ^{99m}Tc174H.64.

A Balb/c nude mouse bearing a FaDu allograft was injected intravenously with 900 μCi (33.3 MBq)/300 μg of ^{99m}Tc174H.64, as described in section 3.2.8.2. A whole-body scintigraphy of the mouse was performed at 24 hours post injection time. The result of this study is presented in Figure 27 as an anterior view of the whole-body image of the Balb/c nude mouse. The whole-body image contains a radioactivity of 1 x 10^5 counts accumulated in 40 minutes time. The 24 hour whole-body scintigraphy of the Balb/c nude mouse shows a high localization of radioactivity in the tumor. Accumulation of radioactivity is also observed in the bladder, kidneys and liver. The uptake of ^{99m}TcMAb by the liver may be due to the non-specific binding of the antibody to the Fc receptors located on hepatocytes (Keenan A.A., Harbert J.C. and Larson S.M., 1985). The high localization of ^{99m}Tc-radioactivity observed in the kidney is probably due to the transchelation of ^{99m}Tc by amino acid residue such as cysteine (Thakur M.L., *et al.*, 1991).

FaDu cell line originated from a human squamous cell carcinoma of the pharynx. Immunohistological staining of the FaDu cells in culture, as reported in section 4.1.1., showed virtually no reactivity with MAb174H.64. However, cytokeratin protein extracted from the same cell type exhibited a high degree of immunoreactivity with MAb174H.64, as reported in section 4.2.1. The whole-body scintigraphy of the balb/c nude

Figure 27 A 24 hour whole-body scintigraphy of 33.3 MBq of ^{99m}Tc174H.64 in a Balb/c nude mouse bearing a FaDu xenograft.



The whole-body image contains a radioactivity of 1.5 x 10^5 counts collected in 40 minutes time.

mouse bearing FaDu xenograft showed distinct localization of the ^{99m}Tc174H.64 in the tumor. The reason why the FaDu cells in culture do not show immunoreactivity with MAb-174H.64 has also been discussed in section 4.2.1. One possibility for this non-immunoreactivity could be due to the non-accessibility of the MAb174H.64 to the intracellular cytokeratin protein. Another reason may be that on repeated growth in culture media some types of squamous carcinoma cells change their cytoskeletal composition and became non-immunoreactive with the antibody. However it is also reported elsewhere that on re-growth in tissue SCC cells regain the lost cytoskeletal characters (Connell N.D. and Rheinwald J.G., 1983). Therefore, even though the FaDu cell in culture showed lack of any immunoreactivity with MAb174H.64, the high localization of the MAb in the FaDu allograft may be as a result of the restoration of the cytokeratin in the tissue. Figure 27 further confirms that ^{99m}Tc labeled MAb174H.64 is capable of providing diagnostically useful scintigrams of SCC tumor sites.

4 8. AN ESTIMATE OF THE RADIATION ABSORBED DOSE TO A HUMAN ADULT FOLLOWING THE I.V. INJECTION OF ^{123}I-174H.64

It is important to determine the radiation energy transmitted to critical organs in order to evaluate the hazard of a radiation exposure or the effect of radiation therapy. The quantity of energy imparted to a defined mass of tissue is termed as radiation absorbed dose. If the absorbed dose is evaluated in terms of MeV of energy delivered to a gram of tissue it is expressed in the conventional units as rads or millirads (1 rad = 100 ergs/g of tissue = 62.5 X 10^6 MeV per gram). In the SI unit where energy is expressed as Joule, the radiation absorbed dose is in Gray (Gy). The conversion factor is 100 rad = 1 Gy.

There has been increasing evidence that radiolabeled MAb deposition within many solid tumor xenografts is not homogeneous (Pimm M.V. and Baldwin R.W., 1986; Vessella R.L., *et al.*, 1988; Sands H., *et al.*, 1985; Kassis A.L., 1992). The knowledge of the distribution pattern of radioactivity within a tumor mass helps to accurately determine the absorbed dose in the tumor. Attempts made to directly measure the locally absorbed radiation dose using miniature implantable thermoluminescent dosimeters (TLD) showed some promising results (Palme D.F., *et al.*, 1991). However, in this calculation an assumption was made that the distribution is uniform over the whole organ.

The average radiation dose to an organ is calculated by determining the total energy deposited in that organ per unit

mass of the organ. The energy deposited in each organ comes from the radioactivity in the organ itself and from that in other organs. Hence, the total dose to a specific organ (D) is the sum of the self dose, D_o, (the dose due to the radioactive material in the organ itself) and the organ-to-organ dose, D_{ts} (the dose to the specific organ due to the radioactive material in all the other organs that have significant contribution) i.e. $D = D_o + D_{ts}$.

The energy deposited in the organ and the organ acting as a target organ, is calculated by considering the energy released in the source organ and the fraction of it which is absorbed in the target organ. The amount of energy released in the organ by any one type of radiation is determined by calculating a) the accumulated activity of radioisotope (À), which requires the fraction of the administered dose in the organ, the effective half life of the radioisotope in that organ and the activity time integral and b) the fraction contribution of the radiation and its average energy (Δ_i) determined from decay scheme analysis. The fraction of energy released by a particular type of radiation that is absorbed in the organ is available from absorbed fraction tables. A scheme for the radiation absorbed dose calculation is presented in the Appendix II. The long and cumbersome calculation necessary to determine the total radiation dose estimate for the various target organs has been simplified by the availability of a computer program.

The internal radiation dose estimate to a human adult following the i.v. injection of ^{123}I-174H.64 is calculated based on MIRD method employing a computer program "MIRDOSE2" (Oak Ridge Laboratories, Tennessee, U.S.A.). More detailed information on the procedure of the MIRDOSE2 method is available in the documentation package for this program and from the report made by Erwin W.D. (Erwin W.D., et al., 1992). Briefly, the isotope of interest is selected and the computer pulls out the decay data for the nuclide. Then the number of source organs and target organs are selected. At this point, the program calculates S-values for all target organs and source organs chosen. The residence times for the source organs are then entered. Residence time = 1.443 X effective half-life (from organ radioactivity clearance curve) X maximum percent injected dose. The computer, using all these data, calculates and prints the radiation dose estimate for each target organ and lists these estimates in mGy/MBq. Table 25 shows the radiation dose estimate to a human adult following the i.v. injection of ^{123}I-174H.64, calculated by this computer program. The following data were taken in to consideration in the calculation of the radiation absorbed dose.

1. The radiation absorbed dose in each organ was calculated on the basis of the % I.D./g tissue of ^{125}I-174H.64 in the DBA mice a bearing KLN-205 tumor, presented in Table 24.
2. The radiation absorbed dose was calculated for an average adult with a body weight of 70 kg injected i.v. with

Table 25 The radiation dose estimate to a human adult following the i.v. injection of ^{123}I-174H.64.

Target Organ	Total residence time in hours	Total dose (mGy/GBq)
Liver	0.70	23.5
Kidney	0.25	36.5
Lungs	0.11	6.3
Stomach	0.53	23.2
Spleen	0.08	23.6
*Testes		10.1
*Ovaries		8.9

*The total absorbed dose estimate for the testes and ovaries were provided by the computer, based on experimental values on phantom studies.

^{123}I-174H.64.

3. The total residence time of radioactivity in each organ was determined making use of the exponential curve of the percent injected dose per gram tissues versus post-injection time (Figure 23).

In the process of radioimmunodiagnosis or radioimmunotherapy, the radiation exposure of non-target organs is a problem which needs to be considered. Table 25 shows the radiation absorbed dose estimates for the various organs of adult human injected i.v. with ^{123}I-174H.64. Based on extrapolation from the mouse animal model studies, the radiation dose estimates for the various organs range from 6.3 mGy/GBq for the lungs to 36.5 mGy/GBq for the kidney. Results reported by other research groups showed that absorbed doses for organs vary with change in the antibody used. Radiation absorbed doses for liver in the range of 1-25 cGy/ MBq was reported (Meredith R.F., *et al.*, 1991; Palme D.F., *et al.*, 1991). The cellular effects of radiation absorbed dose for a wide range of dose-rates were reported by Hall E.J.. and Brenner D.J. In their report they categorized very low dose-rate, 1-100 cGy/day, to cause little or no effect on the growth of cells in culture (Hall E.J. and Brenner D.J., 1991). Our result is in the milli-Gray range and therefore expected to cause minimal damaging effect on the tissues of the organs. Since no early time period distribution results were used, this radiation dose estimate is only a crude approximation.

5. SUMMARY AND CONCLUSIONS.

The main objectives of the present study included:

A) The immunohistological screening of SCC tumor tissues and cell lines for their tissue typing and immunoreactivity with MAb174H.64.

B) The isolation and preliminary characterization of the SCC associated antigen, from the screened SCC tissues or cells, that are immunoreactive with the MAb174H.64.

C) The radiolabeling of MAb174H.64 and the evaluation of the radiochemical stability and immuno-integrity of radiolabeled monoclonal antibody.

D) The investigation of the biodistribution and radioimmuno-scintigraphy of the radiolabeled MAb174H.64 in suitable animal models bearing a SCC tumor.

E) The evaluation of the potential usefulness of the radiolabeled MAb174H.64 in the radio-immuno-scintigraphy of SCC tumor tissue.

Based on these objectives the results and discussions of *in-vitro* and *in-vivo* experiments as presented in the preceding chapters, are summarized below and conclusions are made.

1. Tissues and cell lines from various squamous cell tumor sources were analyzed immuno-histologically in order to screen and select those which were strongly immunoreactive with MAb174H.64. The results revealed that tumor tissues which have a high percentage of their cells as squamous type showed very strong

immunoreactivity with MAb174H.64. This indicates that MAb174H.64 is a sensitive marker for SCC.

2. Cytokeratin proteins were extracted from SCC tissues and cell lines which exhibited strong immunoreactivity with MAb174H.64. SDS-PAGE electrophoresis analyses of cytokeratin protein extracted from bovine SCC tumor tissues showed two distinct bands of cytokeratins, acidic and basic monomers at molecular weight of 58 kd and 64 kd respectively. The 58 kd bovine cytokeratin (56.5 kd human equivalent) is categorized as #10 according to Molls' catalogue. The 64 kd bovine (65.5 human equivalent) is also categorized as #2 and #3 (Moll R.H., *et al.*, 1982). Attempts to quantitatively isolate the monomers were not successful.

3. The immunoreactivity of the MAb174H.64 with cytokeratin protein extracted from SCC tissues and cell lines was determined by an ELISA method of analysis as well as by a competitive binding assay. The results showed that MAb174H.64 is selectively immunoreactive with SCC cytokeratin protein.

4. Monoclonal antibody 174H.64 was radiolabeled with ^{99m}Tc and ^{125}I and the radiochemical stability and the immunoreactivity of the radiolabeled products was determined. The results indicated that the MAb174H.64 was successfully labeled with ^{99m}Tc (370 kBq/ 12 μg of antibody) and ^{125}I (296 kBq/ 6 μg of antibody) with

radiochemical yield of > 98% (labeling efficiency) and with high degree of immunoreactivity.

5. The radiochemical stability and the immunoreactivity of radiolabeled MAb174H.64 subjected to various physical and chemical changes were evaluated. Incubation in human plasma or serum fluids for a period of up to 48 hours showed no significant effect on the radiochemical stability or the immuno-integrity of the radioiodinated or ^{99m}Tc labeled MAb174H.64. However, incubation of the radioiodinated MAb174H.64 in urine showed a significant reduction in both radiochemical stability and immuno-integrity of the radiolabeled MAb174H.64. Exposure of the MAb174H.64 to a very high temperature i.e. more than 50°C, has been shown to reduce the immunoreactivity of the MAb174H.64, most likely due to protein denaturation. Iodogen concentration over 6 μg / 100 μg of protein produced significant effects on the immunological stability of the MAb174H.64. There was no effect detected due to the incubation of the MAb174H.64 in concentrations of stannous chloride solution ranging from 1μg/100μg MAb174H.64 to 50μg/100μg MAb174H.64.

6. The biodistribution of ^{99m}Tc174H.64 performed in Balb/c nude mice bearing a KLN-205 tumor showed twice as much localization of the radiolabeled MAb in the tumor compared to that observed with a control study using ^{125}I-MOPC-21. Another biodistribution study on

^{125}I-174H.64 in DBA mice bearing a KLN-205 tumor showed that the radiolabeled antibody localization in the tumor on a per gram basis was significantly higher than the localization in the other organs except in the blood. The control study using ^{131}I-MOPC-21 showed that the localization of the radiolabeled MAb174H.64 in the tumor was significantly higher than the localization of the control. Both of these results support the selective localization of MAb174H.64 in the SCC tumors.

7. Whole-body scintigraphy of a DBA mouse, bearing a SCC KLN-205 tumor, injected i.v. with ^{125}I-174H.64 was performed at 6 hr, 24 hr, 48 hr and 120 hr post injection time. The results exhibited distinct localization of radioactivity in the SCC tumor. The control study using ^{131}I-MOPC-21 performed in the same way, showed only low level non-specific localization in the tumor. Another whole-body scintigraphy of a Balb/c nude mouse bearing a SCC FaDu allograft was performed using i.v. injection of ^{99m}Tc174H.64. The 24 hour scintigraphy showed a distinct localization of the radiolabeled antibody in the tumor. The results from both investigations indicated that radiolabeled MAb174H.64 specifically localized in SCC tumors grown in the two mouse models.

8. The blood clearance rate of the ^{131}I-174H.64 (IgG) and the ^{125}I-174H.64 $F(ab')_2$ fragment in rabbits were compared. The advantages and disadvantages of the more rapid blood level clearance of the $F(ab')_2$ fragment and the relatively slow blood clearance of the radiolabeled whole-antibody, in scintigraphy studies, were discussed. The radiolabeled fragment provides a lower background radioactivity as well as a lower tumor radioactivity concentration. The whole-antibody, on the other hand produces higher background radioactivity and tumor localization. This is because it clears from the blood at a slower rate and provides a continuous supply of the radiolabeled antibody to the tumor. Therefore the choice of which form of the antibody to use depends on the decision of which quality is more important for a particular study.

The *in-vitro* and *in-vivo* results obtained from the various investigations showed that monoclonal antibody 174H.64 has a promising potential usefulness in the diagnoses of squamous cell carcinoma of various organs. The utilization of this antibody labeled with a proper radioisotope in the treatment of malignant SCC tumor is also a very attractive prospect. Further clinical investigation is recommended to promote its application for human use. At present ^{99m}Tc-labeled MAb174H.64 is undergoing clinical trials with promising results (Baum R., *et al.*, May 1993, Oct. 1993; Baum R., *et al.* submitted for publication, 1993).

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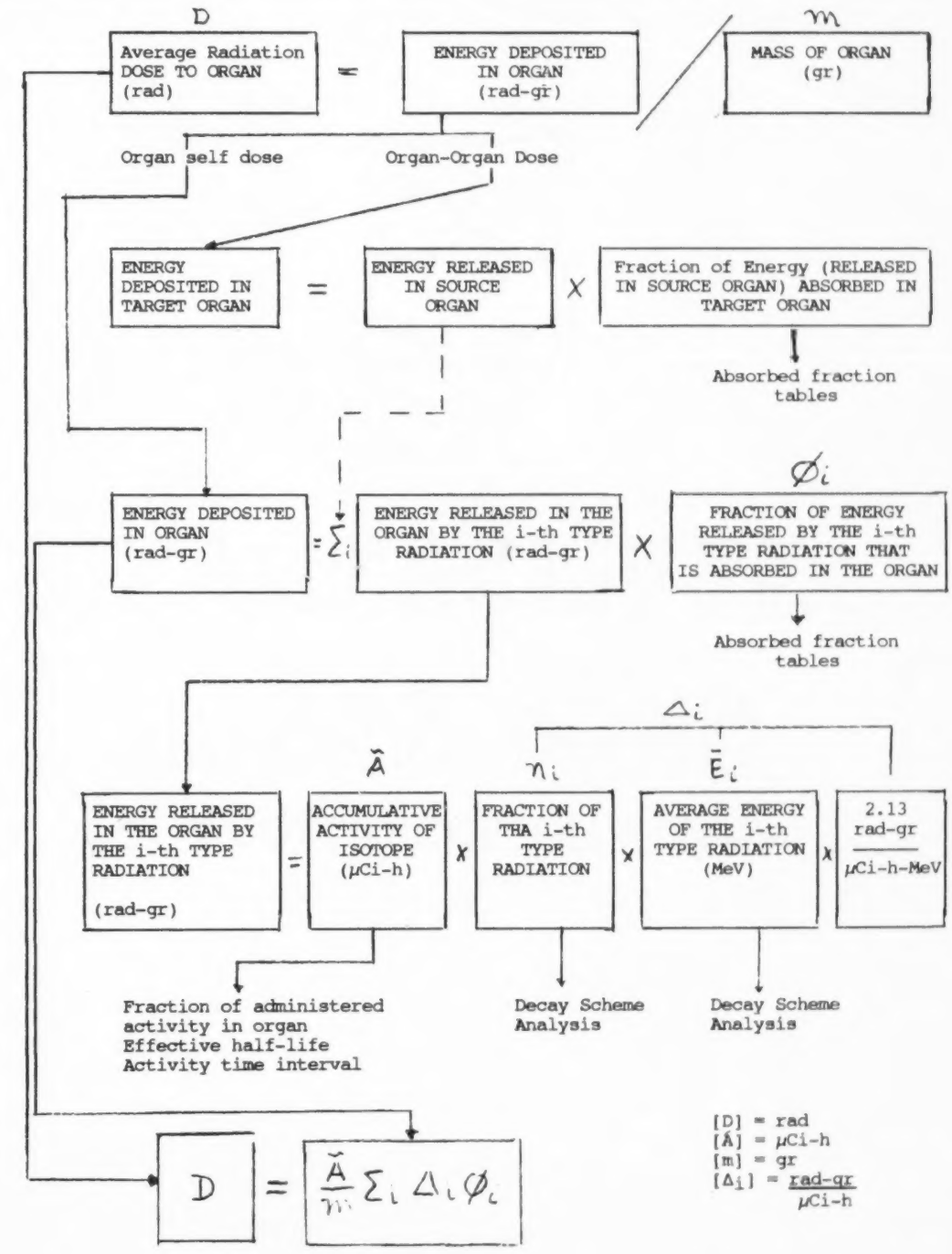
Appendix I. Biodistribution of ^{99m}Tc174H.64 and ^{131}I-MOPC-21 (control) in Balb/C nude mice bearing a KLN-205 allograft.

Organ	^{99m}174H.64 Percent injected dose per gram tissue	^{99m}174H.64 Percent injected dose per organ	^{131}I-MOPC-21 Percent injected dose Per gram tissue	^{131}I-MOPC-21 Percent injected dose Per organ
Blood	4.12 (0.48)	5.47 (0.28)	13.26 (1.71)	18.16 (2.50)
Tissue	4.71 (0.78)	0.24 (0.13)	6.53 (1.83)	0.31 (0.16)
Spleen	2.28 (0.58)	0.33 (0.13)	2.84 (0.26)	0.39 (0.03)
Kidney	8.49 (0.72)	3.96 (0.59)	3.31 (0.26)	1.53 (0.86)
Liver	5.44 (2.42)	8.06 (3.54)	3.89 (0.62)	5.82 (0.57
Lung	1.96 (0.78)	0.32 (0.13)	5.79 (0.81)	0.96 (0.17)
Stomach	0.50 (0.05)	0.25 (0.02)	1.46 (0.15)	0.71 (0.06)
Colon[1]	1.34 (0.22)	1.99 (0.50)	2.14 (0.19)	3.32 (0.32)
Muscle	0.71 (0.31)	- -	1.37 (0.41)	- -

1 Colon is large and small intestine with contents.

The percent injected dose remained in the whole-body at 24 hour post-injection time was 42.6% ±(4.4) of the ^{99m}Tc radioactivity and 70.0% ±(6.8) of the radioiodine.

Appendix II Radiation absorbed dose calculations, flow chart (Adopted from Schlesinger T., 1976).